The Multi-Enzymatic Synthesis of Xylulose 5-Phosphate

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• Abstract •

This thesis illustrates a method for identifying the traits of a multi-enzymatic reaction and defining potential problems early. A systematic approach has been followed and xylulose 5-phosphate synthesis was used as an example. On a commercial level xylulose 5-phosphate is chemically difficult to produce and therefore biocatalysis has been considered as an alternative. A characterisation procedure was developed for this system involving triosephosphate isomerase and transketolase. Particular attention was given to the mechanics of running the system starting from dihydroxyacetone phosphate and glyceraldehyde 3-phosphate rather than fructose 1,6 bisphosphate. The results were used to indicate the process limits for this model system and the supply of glyceraldehyde 3- phosphate was shown to be the key bottleneck in the production. These data were reported in the form of operating maps / windows for the process. The scientific information gathered on the multi-enzymatic system together with key engineering concepts were used to synthesise alternative process routes for the production of xylulose 5-phosphate. These processes were screened based on their attributes and a logical scoring process was developed. This scoring considered the economics, thermodynamics, kinetics and the complexity of each process for the selection and operation of the most scalable option. As a result, unattractive or unviable biocatalytic systems were eliminated early. The best process options for production were identified and tested. These process selection techniques would appear to be applicable to a broad range of biocatalytic systems and could prove vital in ensuring industrial success at an early stage of process development.

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• Abbreviations •

CER	carbon dioxide evolution rate
DCW	dry cell weight
DHAP	dihydroxyacetone phosphate
DOT	dissolved oxygen tension
Fru 1,6 BP	D-fructose 1,6 bisphosphate
FruA	D-fructose 1,6-bisphosphate aldolase
G3P	D-glyceraldehyde 3-phosphate
HPA	hydroxypyruvate
HPLC	high performance liquid chromatography
HTS	high throughput screening
ISPR	*in-situ* product recovery
n	estimated value of enzyme/substrate
NMR	nuclear magnetic resonance
OD	optical density
OUR	oxygen uptake rate
R5P	ribulose 5-phosphate
RO	reverse osmosis
STR	stirred tank reactor
Tk	transketolase
Tki	impure (crude) transketolase
Tkp	purified transketolase
TPI	triosephosphate isomerase
TPP	thiamine pyrophosphate
Tris	tris-(hydroxymethyl) aminomethane
x	Estimated value of substrate / enzyme
X5P	D-xylulose 5-phosphate
$Y_{[P]/[S]}$	yield of product from substrate
$Y_{[P]/[E]}$	yield of product from enzyme

• Nomenclature •

[E]	biocatalyst concentration in the STR (gl^{-1})
[P]	product concentration in the STR (mM)
[S]	substrate concentration in the STR (mM)
kat	Enzyme activity: amount of product formed ($mM\ min^{-1}$)
K_m	Michaelis-Menten parameter (mM)
P	Productivity ($gl^{-1}hr^{-1}$)
s	Specific activity ($U\ mg^{-1}$)
STY	space-time yield ($gl^{-1}hr^{-1}$)
t	time (minutes)
U	units of enzyme activity ($\mu molmin^{-1}$)

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1 Introduction

1.1 Multi-enzymatic synthesis

The work presented in this thesis is concerned with solving practical issues involved in running a reaction with multiple substrates and enzymes. Systems involving more than one enzyme are rather complex in nature. They are difficult to implement in industry. There is little literature to draw upon in order to fully understand these processes. Figure 1.1 highlights the issues that were addressed in this investigation for the analysis of a model multi-enzymatic synthesis.

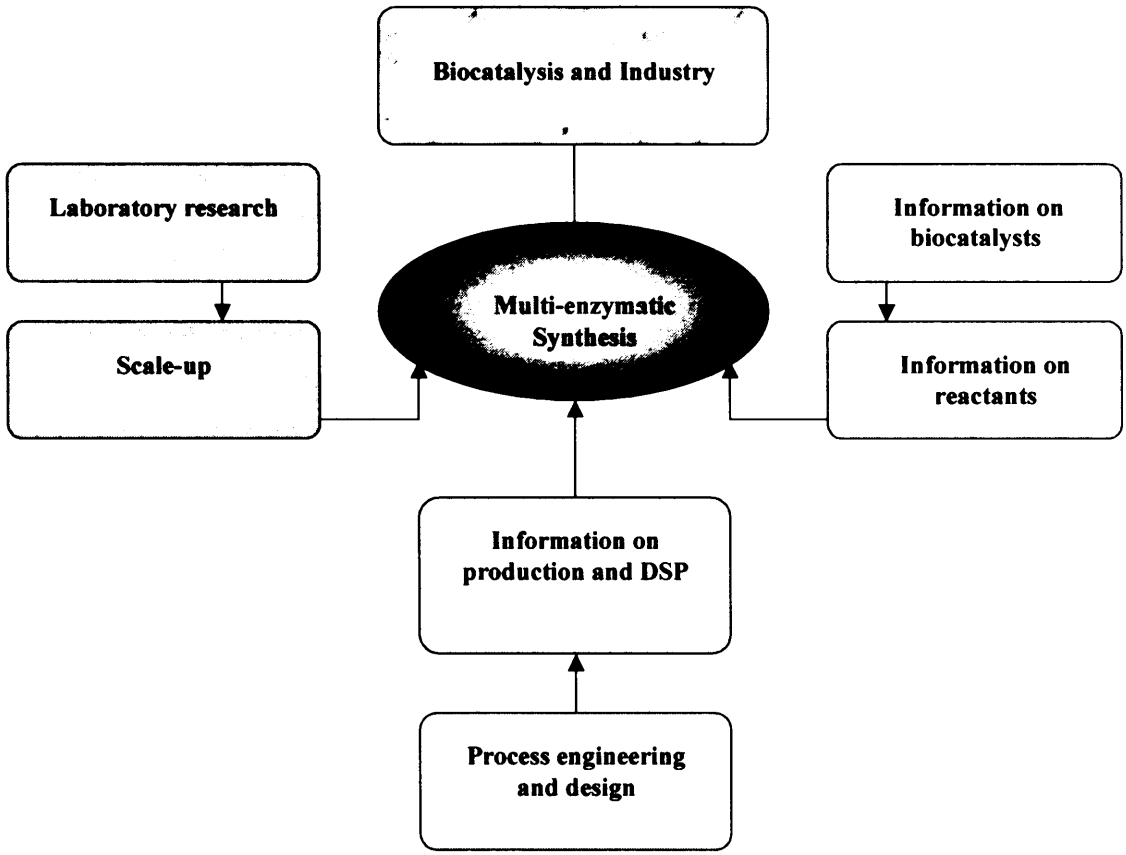


Figure 1.1 Investigating a multi-enzymatic synthesis and the routes that can be considered.

1.1.1 Biocatalysis and industry

Biocatalysis is now a standard technology in the fine chemicals industry. The number of biotransformation processes running on a commercial scale has been steadily increasing in recent years. The number of biotransformation processes which have already been developed to industrial scale has increased and 134 were reported in 2002. Reports have been published on the technical criteria that biotransformation processes have to meet in order to be of commercial significance. Chemically driven components are reaching the market at an increasing speed. To compete at a commercial level biocatalytic processes need to be developed rapidly. One of the major requirements for biotransformation processes in industry is fast process development for industrial use (Straathof *et al.*, 2002).

There are a number of publications helping to build a strong framework of parameters and guidelines for biocatalytic process development. With advances in analytical techniques and the use of information technology biotransformation processes are being fully investigated at an ever-increasing rate. Computer modelling and micro scale techniques allow detailed studies based on very small amounts of components. Vast numbers of processes can be analysed very quickly with great accuracy and detail. To fully benefit from the technologies available there must be a logical progression from process design, to rapid biocatalyst development and production, to process selection and realisation. More studies are being conducted with this aim, which could eventually help a whole variety of biotransformations still in the preliminary stages of research gain commercial success and application to industry (Woodley and Lilly, 1996).

1.1.2 The analysis of multi-enzymatic systems

Multi-enzymatic systems are analysed either in whole cell systems or in groups of isolated enzymes and many are still in the exploratory phase. They could play a major role in industry as they generally lead to natural compounds and their derivatives, which are difficult to produce chemically. So far the studies have been important to biological science but due to their complexity very few have been tried at large scale or for industrial use. Mosbach and co-workers pioneered some of the more detailed process studies on isolated multi-enzymatic systems. With extensive research carried out on immobilised systems with studies on pH-activity profiles of

an immobilized two-enzyme system (Gestrelius *et al.*, 1972) and three enzyme systems (Mattiasson and Mosbach, 1971). The work published by Mosbach and Mattiasson (1976) also details work on multi step enzyme systems. The findings of this group were later applied to the development of multi-enzymatic thermistor devices and the determination of enzyme activities with the enzyme thermistor unit (Danielsson and Mosbach, 1979). Methods of calculating time-course behaviour of multi-enzyme systems from the enzymatic rate equations have also been proposed (Rhoads *et al.*, 1968) using computers and mathematical equations for simulating enzyme systems. Computer programs have been devised with equations representing the behavior of enzymes (Straathof *et al.*, 2001). These process simulations using equations have so far been slow and often inaccurate in predicting the behavior of enzymes (Willeman *et al.*, 2000). More recently progress has been made on analysis of the kinetics in an isolated multi-enzymatic system. Ivanetich and co workers published work on the desaturation of linoleic acid in hepatic microsomes. Firstly improvements were made on assay techniques. Using HPLC analysis they were able to study the kinetics of the desaturase in a multi-enzymatic system. By gathering experimental data and the use of mathematical modelling kinetic constants (Km, Vmax) were calculated for Acyl-CoA Synthetase, desaturase and lysolecithin acyltransferase. The study helps highlight major issues relating to the system. It was found that the presence of endogenous linoleic acid affects directly the total substrate concentration and in turn specific activity and rate of product formation. The work also raised new questions on the extent to which acyl-CoA hydrolase and lysophospholipid acyltransferase compete for fatty acyl-CoA substrate. In conclusion this type of study paved the way for delving further into how biocatalysts work together (Ivanetich *et al.*, 1996).

Bieberich and co workers also proposed a theory for multi-enzyme kinetic analysis (MEKA) in a whole cell system. The synthesis of gangliosides sequentially by a series of glycosyltransferases acting in parallel biosynthetic pathways was studied again by calculating Vmax and Km values of each enzyme. This analysis was implemented based on known facts about the system and a series of assumptions. However the MEKA neglected the degradation of gangliosides and product inhibition as studies with NG108-15 cells (Murine neuroblastoma X rat glioma) in short-term labelling experiments. In this system two enzymes use the same ganglioside pool of substrate and, in turn are competitive towards each other. It was

highlighted that MEKA can be applied to other biosynthetic pathways and provides the advantage of analysing kinetic data with intact cells or tissue samples (Bieberich and Yu, 1999). It is clear that the development of such models can eventually be useful in the analysis of cells/tissue such as those in the nervous system and brain for example in sphingolipid storage disorders which may result from both deficiencies of degradative as well as biosynthetic enzymes.

1.1.3 Multi-enzymatic systems and industry

Knowledge on multi-enzymatic systems can be useful in industry as it can provide means of reducing process costs and improving process yields. Figure 1.2 shows an example of how this may be possible. This is where an expensive, high value, low concentration substrate (B) often limits the use of a process where C is the product and Y is the biocatalyst. In this case it may be possible to introduce a cheaper substrate (A) and with the aid of a second enzyme (X) produce the expensive component in the reactor. This seems a relatively simple idea but it is practically difficult to implement. To run the system successfully it is vital that the enzymes work well together. As is often the case there are constraints to consider with each biocatalyst. By carrying out key experiments and gathering important data it is possible to run such a system successfully. Many systems found in nature use a variety of pathways to produce the same product and there is no reason why the same principle cannot be applied to industrial biocatalysis with the use of multiple isolated enzymes.

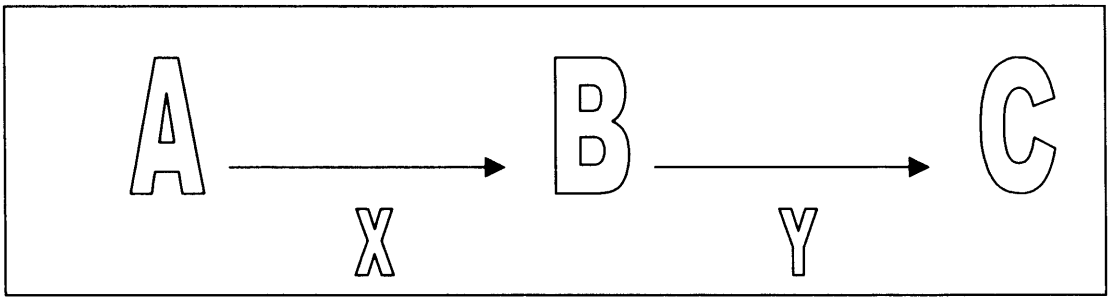


Figure 1.2 The logic of adding an enzyme to a biocatalytic system. Product C is prepared using a high value substrate (B) and enzyme Y. An alternative route is introducing a cheaper substrate (A) with the addition of a second biocatalyst (X).

On this basis the Fessner groups work has been important as most of the research has focused on the synthesis of natural products using aldolases and related enzymes (Fessner and Helaine, 2001). The work shows a number of isolated enzymes for use in C-C bond formation and their potential for application to asymmetric synthesis (Fessner, 1998). More importantly the systems are often multi-enzymatic and closely related to naturally occurring systems such as the pentose phosphate pathway and glycolysis (Hixon *et al.*, 1996). The natural strategy for the glycosidase-assisted synthesis is an example of this work (Ruiz *et al.*, 2001) as well as the multi-enzymatic synthesis of -xylulose 5-phosphate (Zimmermann *et al.*, 1999). From the examples in literature it is clear that the development of a multi-enzymatic system requires a good understanding of biocatalysis.

1.1.4 Biocatalysis background

Biocatalysis is the preparation of complex molecules using enzymes. An annual average of approximately 600 scientific original papers have been published since the late 1980s in this interdisciplinary field between organic chemistry and biotechnology (Faber *et al.*, 2000). Today biocatalysis is a standard technology for the production of fine chemicals and the total number of biotransformation processes that are being carried out on an industrial scale has grown rapidly. Approximately 140 biocatalytic processes were reported in 2002 where 25% involved the production of carbohydrates and chiral compounds (Straathof *et al.*, 2002).

1.1.4.1 Biocatalysts

The pharmaceutical industry relies increasingly on biological catalysts for the production of enantiomerically pure compounds. There have been a number of in-depth reports on the production of pharmaceuticals using biocatalysts (Turner, 1995). Biocatalysts are affected by pH, temperature and concentration levels of reaction components. They require water for operation and they are rarely endo- or exothermic. Usually they are affected by product and reactant inhibition and

toxicity. Biocatalysts are made of one or more enzymes. Enzymes are relatively large molecules with high molecular weight, ranging from 20,000 to several million. Enzymes have their own natural structure. This structure is essential for maintaining activity. Enzymes can increase the rate of reactions as much as 10^{14} – fold therefore they are used in the chemical and pharmaceutical industries as they can result in better kinetics and in some cases improved thermodynamics. One of their main advantage is to lower the activation energy of a reaction (Figure 1.3).

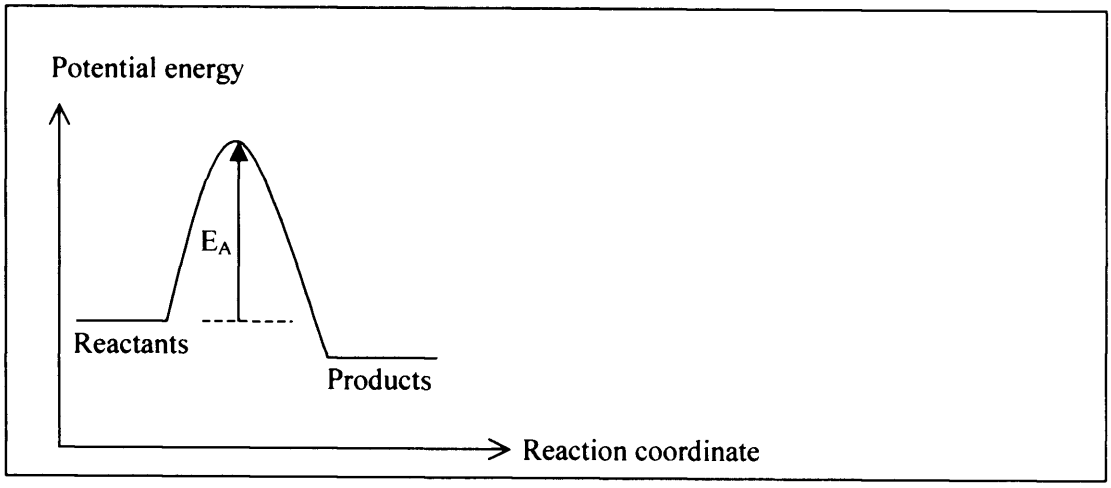


Figure 1.3 Activation energy E_A.

Hence, it is possible to work at a lower temperature (Atkins, 1994). Biocatalysts have some remarkable characteristics, which give them advantages over chemical catalysts. Biocatalysts can be used under mild conditions, thereby the problems of substrate or product decomposition and other side reactions are minimised. More importantly, enzymes from micro-organisms can often carry out in one step a transformation that may require a number of chemical steps, often to high enantiomeric purity. The ability of enzymes to distinguish between enantiomers is perhaps their most valuable attribute. Many pharmaceutical companies have switched to the production of single enantiomers (Cheetham, 1994). For the chemical and pharmaceutical industries the significance of enzymes and their industrial potential is increasing (Dordick and Clark, 2002).

1.1.5 Enzyme kinetics

Enzymatic reactions have been characterised using various models. The Michaelis-Menten model is the most well known description of the enzyme kinetics. It describes the interaction of the enzyme with the substrate. However it should be noted that Michaelis-Menten kinetics are only applicable to simple one-substrate systems and therefore not directly applicable to many systems (Atkins, 1994).

The Enzyme (E) and its substrate (S) bind together to form the activated intermediate (ES) which breaks down to release the enzyme and products (P&Q):

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} ES \qquad (1)$$

$$ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P + Q \qquad (2)$$

Two major assumptions have to be made in the treatment of enzyme kinetics:

- The first equilibrium between the enzyme and its substrate is rapidly established.
- The driving force to break down the enzyme substrate complex (ES) into products (P&Q) is very large.

Therefore the condition is that $k_1 > k_{-1} > k_{-2}$ where K is the dissociation constant for the enzyme-substrate complex.

$$K = \frac{k_{-1}}{k_2} = \frac{[E][S]}{[ES]} \Rightarrow [ES] = \frac{[E][S]}{K} \qquad (3)$$

Rate of reaction = $k_2[ES]$ (4)

The first order rate constant k_2 is the catalytic rate constant for the enzyme often called the turnover number or k_{cat}. The fraction of the initial enzyme concentration E_0 present is:

$$ES = \frac{[S]}{K + [S]} \quad \text{since } [S] >> [E_0], \text{ hence:} \qquad (5)$$

$$\text{Rate of reaction (v)} = \frac{k_2[E_0][S]}{K + [S]} = \frac{V_{max}[S]}{K + [S]} \qquad (6)$$

Where [S] >> [E][S]
Figure 1.4 is plotted to show the rate of reaction as a function of substrate concentration [S]. This is a hyperbolic function for which [S] = K when $v = \frac{V_{max}}{2}$.

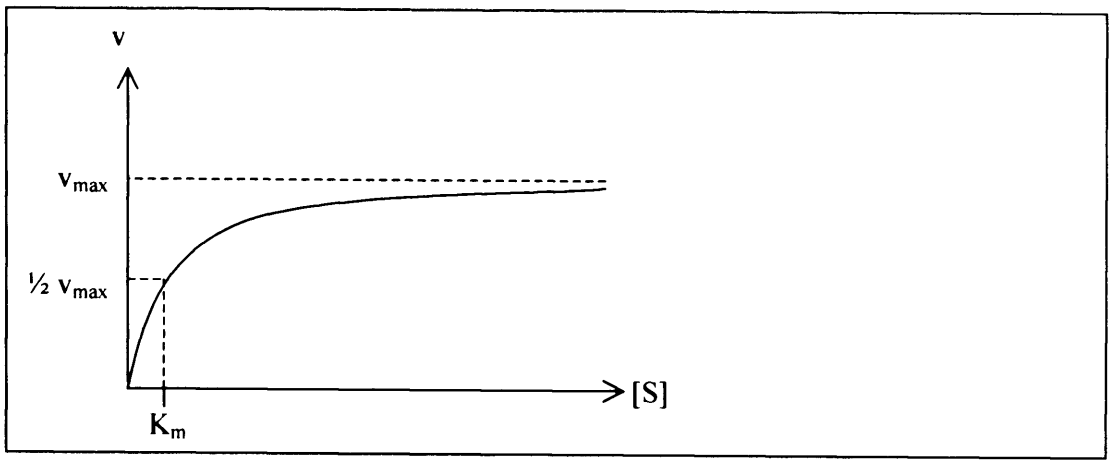


Figure 1.4 Rate of reaction as a function of substrate concentration [S] for Michaelis Menton kinetics.

K is termed K_m the Michaelis constant. Only if k_1 & $k_{-1}>k_2$ does it equal the dissociation constant for the enzyme-substrate complex.

If $[ES] = \frac{[E][S]}{K_m}$, then: (7)

$$v = \frac{k_{cat}}{K_m}[E][S] \quad (8)$$

where the second order rate constant k_{cat}/K_m describes the rate of reaction in terms of the free enzyme and free substrate concentrations. To obtain a linear plot, equation (4) can be inverted. This inversion results in the Lineweaver-Burk plot (Atkins, 1994)

$$\frac{1}{v} = \frac{Km}{[S]} \cdot \frac{1}{v_{max}} + \frac{1}{v_{max}} \qquad (9)$$

A plot of 1/v against 1/[S] shows a straight line with intercepts of $-1/K_m$ and $1/V_{max}$ (Figure 1.5).

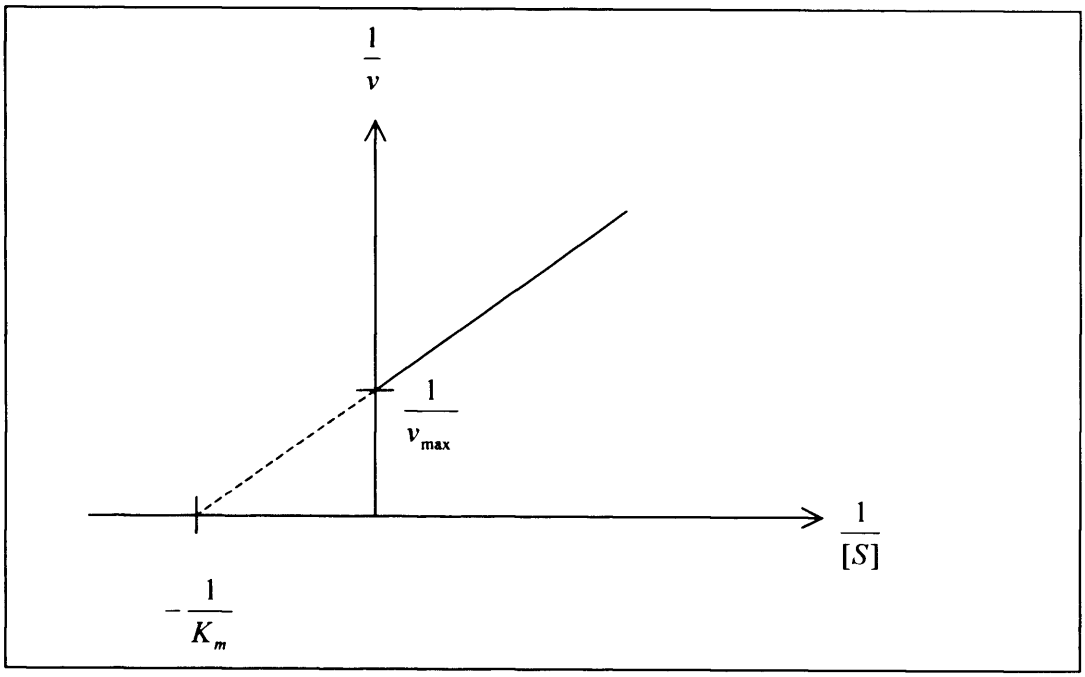


Figure 1.5 Lineweaver-Burk plot (Atkins, 1994).

The kinetics is undoubtedly different in multi substrate systems. The transketolase reaction is an example where the active site is not large enough to accommodate both substrates simultaneously and it is therefore likely that the reaction mechanism follows a ping pong bi bi type mechanism (Lindqvist *et al.*, 1992).

The ping pong bi bi (one on, one off; one on one off) can be described by the schematic in Figure 1.5.

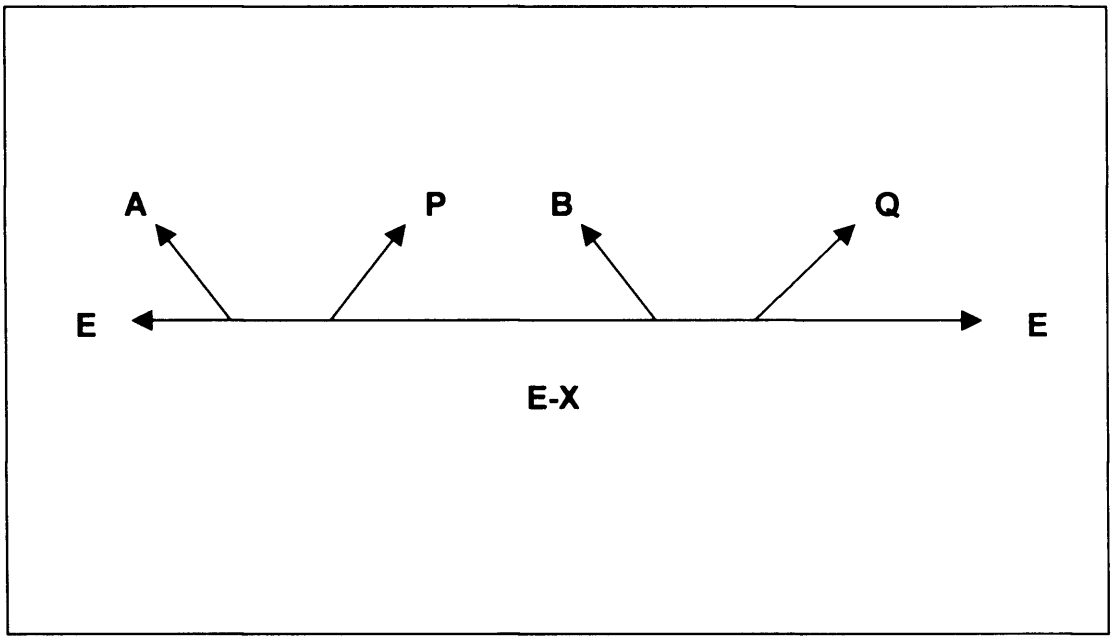


Figure 1.6 Ping Pong Bi Bi kinetic mechanism there is a modified enzyme intermediate (E-X) where substrate A and B result in products P and Q (Atkins, 1994).

In Ping Pong mechanisms (double displacement reactions) one or more product is released before all of the substrates are bound as viewed in the schematic in Figure 1.7.

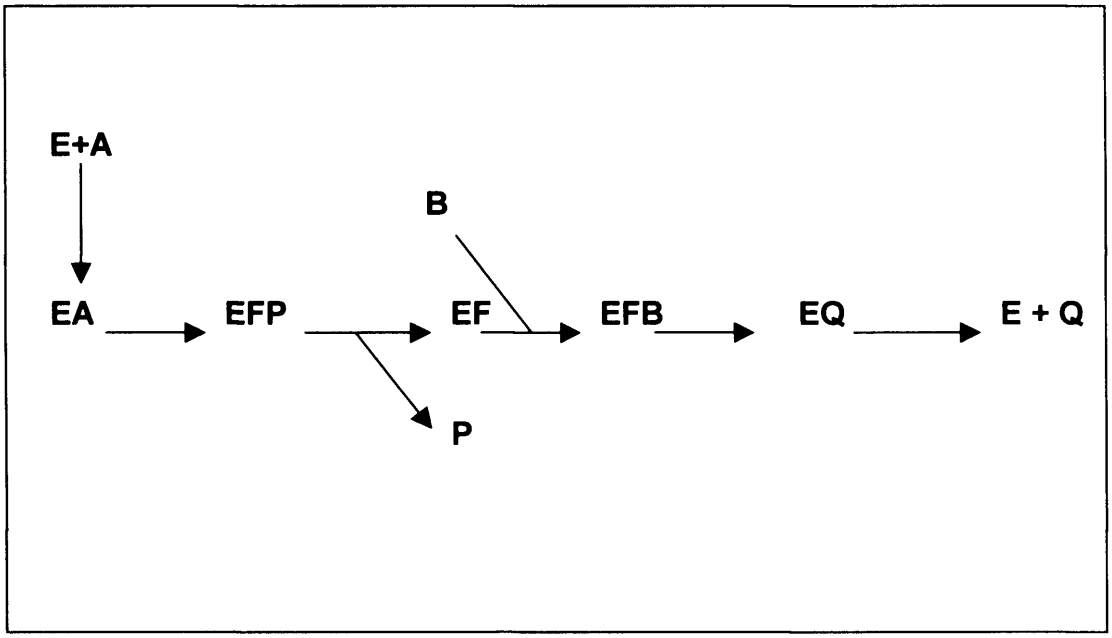


Figure 1.7 Ping Pong Bi Bi kinetic mechanism where one product (P) is released before all of the substrates (A and B) are bound.

Therefore the Ping Pong mechanism can be described by the following equation (Atkins, 1994):

$$\frac{1}{v_0} = \frac{Km^A}{v_{max}[A]} + \frac{Km^B}{v_{max}[B]} + \frac{1}{v_{max}} \qquad (10)$$

1.1.6 Carbon-carbon bond synthesis

A remaining unsolved problem of catalytic asymmetric synthesis is the stereoselective carbon-carbon bond formation in water. Often in chemistry the donor species are incompatible with the aqueous system and cause undesired cross-reactions. The formation of novel C-C bonds is a key need in chemical synthesis consequently biocatalytic processes have gained increasing attention as they catalyse the asymmetric C-C bond formation in water (Faber and Patel, 2000). In many cases new centres of chirality are generated by this means. In this respect biocatalysis is a tool where enantioselectivity is introduced by the asymmetric character of the enzyme active site (Griengl *et al.*, 2000).

1.1.7 TPP dependent enzymes

The synthetic potential of TPP dependent enzymes has been described by Sprenger and Pohl (1999). In the field of carbohydrate chemistry, the inherent multifunctionality of sugars is an enormous challenge for organic chemists who have to approach carbohydrate syntheses with protective groups in order to prevent undesired reactions of the various hydroxyl-, keto-, or phosphate groups. So far mainly lyases and aldolases have been used to synthesise complex sugars, sugar analogues and other biologically important natural compounds. TPP dependent enzymes include the potential for both breaking and forming carbon-carbon bonds in the laboratory and have been used as catalysts in chemoenzymatic synthesis. The spectrum of reactions catalysed by these enzymes encompasses nonoxidative decarboxylations of α-keto acid, caboligation, α-keto acid decarboxylases, acetolactate synthase as well as the cleavage of C-C bonds. All enzymes have in

common a TPP bound active aldehyde intermediate formed by decarboxylation or by transfer from a suitable donor compound. Transketolase is an example of the latter where xylose-5-phosphate is the natural donor compound (Sprenger and Pohl, 1999). Figure 1.8 illustrates the transketolase reaction in the pentose phosphate pathway.

1.2 Transketolase background

1.2.1 Transketolase

Transketolase contains vitamin B1 derived TPP as a prosthetic group and requires Mg^{++} ions for catalytic activity. The enzyme has been isolated from a number of eukaryote sources such as *E. coli* as well as baker's yeast, rat liver , pig liver, human erythrocytes and spinach. *E. coli* transketolase (EC:2.2.1.1) has two subunits. The molecular weight of transketolase is approximately 70000 Daltons per subunit and is important in the carbon/carbohydrate metabolism of all cells linking the pentose phosphate pathway with glycolysis. The enzyme was first reported in a study on the mechanism of ribulose 5-phosphate and ribose 5-phosphate (de la Haba and Racker, 1952) as well as by Horecker and co workers (Horecker *et al.*, 1957). Clark Gubler described the physiological functions and the mechanism of transketolase action as related to the pentose phosphate pathway highlighting the key role of transketolase for the metabolism of glucose 6-phosphate (Bisswanger and Ullrich, 1991). Figure 1.8 describes the pentose phosphate pathway based on the work by the Horecker group (Horecker *et al.*, 1992).

In recent years there has been substantial progress in developing transketolase as a catalyst for asymmetric carbon-carbon bond synthesis. The *E. coli* enzyme has been overexpressed and is now available in substantial quantities (Woodley *et al.*, 1996). Much effort has gone into defining the problems associated with operating the reaction at scale in certain cases generic solutions have been identified (Hobbs *et al.*, 1996). The range of potential targets accessible via transketolase catalysed reactions has been expanded by widening the pool of successful substrates and also exploring further synthetic applications of the products such as the synthesis of monosaccharides and heptulose analogues (Andre *et al.*, 1998). The synthesis of

the compounds found in the pentose phosphate pathway such as sedoheptulose has also been described (Dalmas and Demuynke, 1993).

The mechanism of the reaction with regards to the involvement of TPP (Figure 1.13) has been of great interest (Booth and Nixon, 1993) describing the preparation of the active holo enzyme using TPP (Brocklebank *et al.*, 1999). Understanding the transketolase mechanism of action has been aided by X-ray and mutagenesis studies (Littlechild *et al.*, 1995) leading to the possibility of redesigning the enzyme in a directed manner to improve certain desired characteristics. Kinetic studies comparing the kinetic properties of different sources of the enzyme (Masri *et al.*, 1988) have been important. More recently the kinetics have also been described in one-substrate reactions (Bykova *et al.*, 2001). The inhibition mechanism of transketolase (Gorbach, 1980) and the influence of substrates on its conformation (Kovina, 2000) have also been studied.

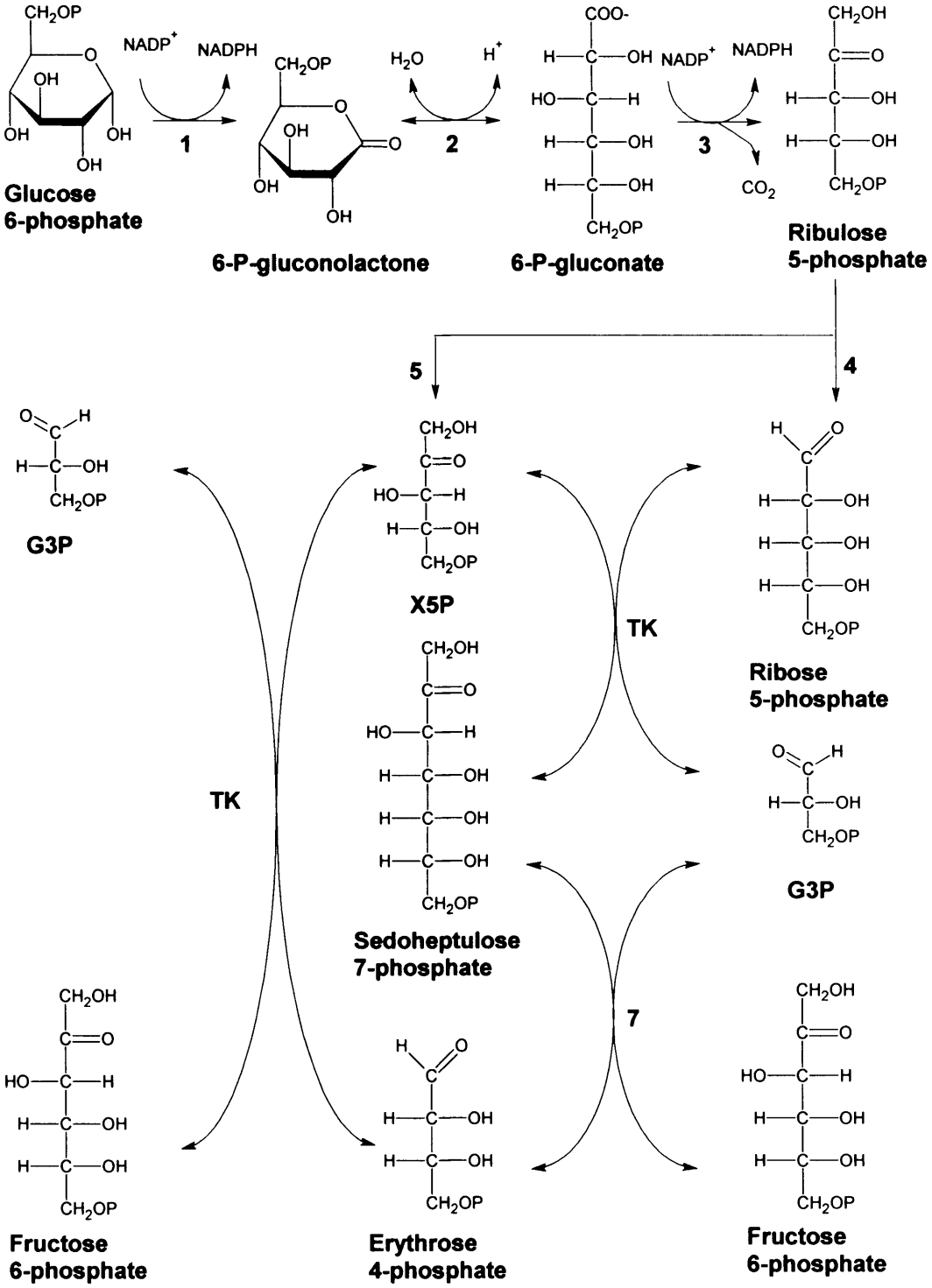


Figure 1.8 The pentose phosphate pathway (modified from Horecker *et al.*, 1992). Showing the metabolism of glucose 6-phosphate by glucose phosphate dehydrogenase (1), 6-phospho-glucono-lactonase (2), pospho-gluconate dehyrogenase (3), ribulose 5-phosphate isomerase (4), ribulose 5-phosphate epimerase (5), transketolase (TK) and transkaldolase (7).

Structural investigations on transketolase have provided the first glimpses of how the enzyme operates. There have been studies on transketolase from bakers yeast and local two-fold symmetry axis has been established, identifying the exact dimensional calculations for the two subunits of the enzyme (Schneider *et al.*, 1992). Bisswanger and Ullrich (1991) published a comprehensive review of the biochemistry and physiology of thiamine dependent enzymes indicating that the TK molecule consists of two identical subunits and has two active centres with essentially the same catalytic activity (Bykova *et al.*, 2000). Crystals of *E. coli* transketolase have grown at pH 6.5 in ammonium sulphate with PIPES buffer and the structure was solved using molecular replacement on the yeast transketolase coordinates. The active site residues and fold is now conserved in both types of enzyme as reported by Schneider and co-workers (Bisswanger and Ullrich, 1991). The dimer has 2 cofactor binding sites (C2 symmetrical structure) (Lindqvist *et al.*, 1992) and therefore 2 identical active sites (Schneider *et al.*, 1993). Figure 1.9 shows the crystal structure of *E. coli* transketolase obtained by X-ray diffraction (Littlechild *et al.*, 1995) is now published in the protein data bank (PDB) downloadable from www.rcsb.org/pdb with PDB ID 1QGD.

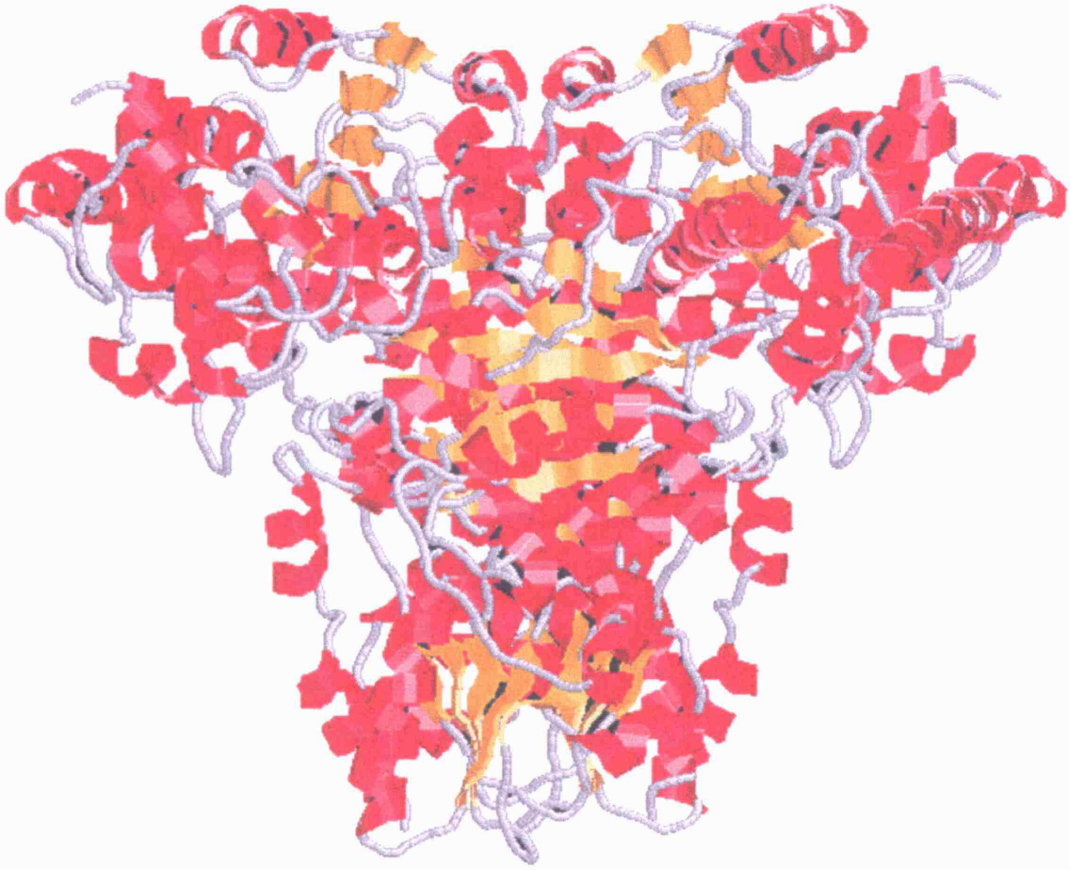


Figure 1.9 The crystal structure of *E. coli* transketolase. This image was created using RasWin Molecular Graphics V 2.6 based on the PDB data available (Isopuv *et al.*, 2003).

1.2.2 Genetics and strain development

Much of the research on transketolase is carried out using the *E. coli* source. This is due to low activities associated with commercially available yeast transketolase and the relatively low yield of preparations from spinach leaves (Demuynck *et al.*, 1991). A rich bacterial source of transketolase opened up the possibility of higher yields from recombinant strains for studies on chemoenzymatic synthesis. The gene for transketolase from *E. coli* has been cloned and in a report by Sprenger and co workers (1995), recombinant *E. coli* K-12 strains over expressed Tk activity. One example was the pGSJ427 Construct of a 4.5 kb insert cloned into a pUC19 vector. The protein purification and properties of the enzyme from the recombinant strain have been described (Sprenger, 1995).

French and Ward (1995) also subcloned the *E. coli* Tk gene into high copy number vectors. This was done by excising a 5kb *Bam*HI fragment from a low copy number vector (pKD112A) and subcloning it into the high copy number *E.coli* expression vector pUC18 carried out in both orientations with respect to the *lacZ* promoter. The 5kb *Bam*HI fragment containing the Tk gene was again excised from one of the resulting constructs (pQR182) and cloned into pBGS18 forming constructs PQR700 and PQR 701. Later construct pQR 711 was created to improve the efficiency of Tk expression. Firstly segments were removed from the *Bam*HI fragment. The remaining Tk fragment was amplified by PCR and a 2.2 kb fragment was cloned into PCRscript SK(+) in both orientations with respect to *lac* promoter forming constructs pQR711 and pQR706 which showed high yields of 168mgL^{-1} culture (23% of total cell protein). Figure 1.10 shows the construction of the transketolase recombinant plasmids by French and Ward (1995) and describes the yields at each stage. Construct JM107/pQR711 was used in this research. This construct was shown to have a specific activity of 6.25 Umg^{-1} in shake flask cultures based on the enzyme linked Tk assay (Figure 1.16) (French and Ward, 1995). The availability of the cloned transketolase gene on a high copy number vector elevated activities in crude extracts and consequently opened up the possibility of characterising the enzymes properties. In fed batch fermentations up to 4gL^{-1} of soluble intracellular transketolase was produced at high levels representing 43% of the total cell protein (Thomas and Woodley, 1998).

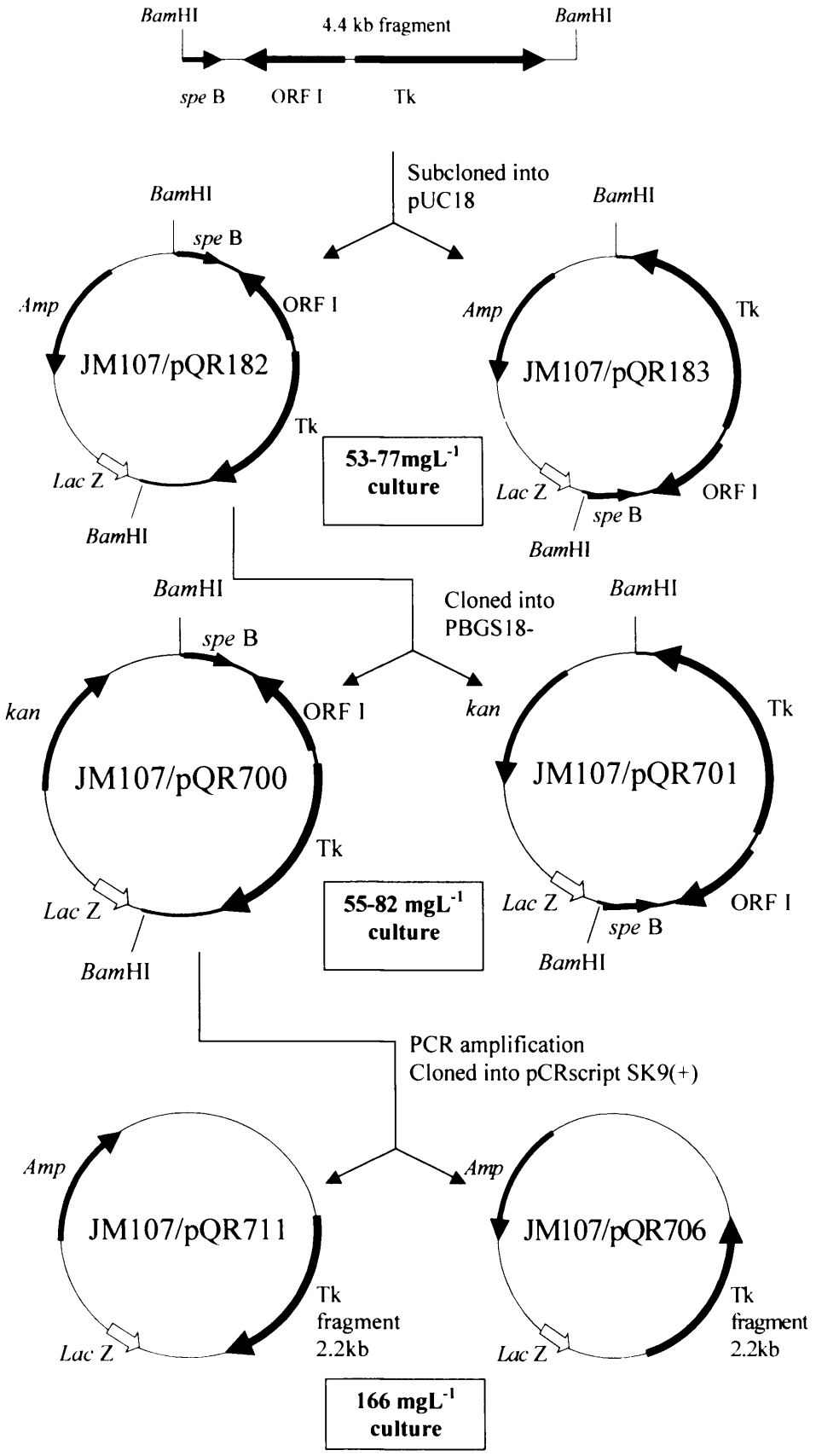


Figure 1.10 The construction of *E. coli* recombinants expressing transketolase (mg L^{-1} culture) as described by French and Ward, 1995.

1.2.3 Large-scale enzyme production and purification

Using the *E. coli* strain JM107/pQR700, Hobbs *et al.*, 1996 have reported a study on the large scale production and recovery of transketolase. Up to 1000 L production process was implemented using a glycerol feeding strategy to obtain a broth with improved cell density (20 g l^{-1} DCW). This was shown to have a transketolase activity of 232 U ml^{-1} and a specific activity of 26.4 U mg^{-1} using a linked enzyme NADH assay (Hobbs *et al.*, 1996). After the fed-batch fermentation a downstream processing scheme was implemented which gave a bacterial cell paste resuspended in 10 mM phosphate buffer, pH 7.6 which could be stored at –20 °C for at least 3 months without loss in activity.

1.2.4 Enantioselectivity

Transketolase naturally catalyses the transfer of a two carbon ketol moiety (Figure 1.11) where the nucleophile (boxed) is transferred to the acceptor aldehyde (eletrophile) forming a carbon-carbon bond stereospecifically. The reaction is reversible in its classical form. Although not its natural function, in the laboratory *E. coli* transketolase also catalyses the condensation of a range of aldehydes with hydroxypyrvate. Particularly those containing an α-hydroxyl group in the D-configuration. Morris and co workers determined the high stereoselectivity for the R-enantiomer. It was shown that however with L-Glyceraldehyde there is essentially no reaction whereas D-glyceraldehyde reacts at approximately the same rate as achiral substrate glycolaldehyde. In this in-depth study a series of aldehydes were applied to transketolase for production with yields of up to 76% (Morris *et al.*, 1996).

1.2.5 The multi-enzymatic system

Up to now research has mainly concentrated on transketolase individually. It is important not to view transketolase as a solitary enzyme. It is clear when simply looking at the role of transketolase in the cell more closely that transketolase works in unison with other enzymes in the pentose phosphate pathway (Figure 1.8). Calvin and Basshan (1962) have shown that ribulose 1,5 bisphosphate is the intermediate acceptor for carbon dioxide in the CO_2 fixation reaction of photosynthesis and have formulated the reductive pentose phosphate pathway most

commonly known as the Calvin cycle. It is apparent from the report that transketolase plays a key role in this cycle, which accomplishes the fixation of CO_2 to form carbohydrates with the regeneration of the starting material ribulose 5-phosphate. Three ATPs are used up for each CO_2 fixed, whereas, in the oxidation pathway no ATP is involved except in the initial phosphorylation of glucose 6-phosphate. Sections of these metabolic pathways have been subject to scientific research since their discovery particularly in biocatalytic transformations using C-C bond forming enzymes (Takayama *et al.*, 1997). Understanding these metabolic pathways has been beneficial for the development of aromatic compounds (Lu and Liao, 1996). Figure 1.11 highlights the classical and natural transketolase pathway. Transketolase *in vivo* catalyses the key conversion of ribose-5-phosphate and xylulose 5-phosphate (X5P) to glyceraldehydes 3-phosphate (G3P) and seduheptulose 7-phosphate (Horecker, 1962). This reaction can also be viewed in the classical pentose phosphate pathway (Figure 1.8).

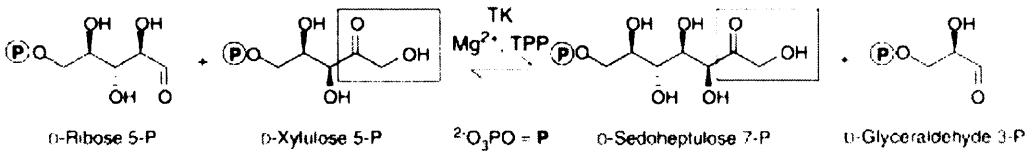


Figure 1.11 The classical transketolase reaction

Many in-depth studies in this field have been carried out on aldolases. Of the several enzymes that catalyse C-C bond formation, aldolases are present in all organisms and belong to the class of lyases. There are over thirty known aldolases most of them catalysing the reversible aldol addition of a ketone to an aldehyde acceptor (Arth and Fessner, 1997). Type I aldolases are found mainly in animals and higher plants (Gustavo, 2000). Type II aldolases occur in microorganisms and are in general more stable. In the type I aldolases the lysine residue forms a Schiff

base with the donor, which then adds stereospecifically to the acceptor (Hixon *et al.*, 1996). Type II aldolases use Zn^{++} as a cofactor (Gustavo, 2000).

FruA from rabbit muscle is an example of a type I aldolase (Schoevaart *et al.*,1999). All type I aldolases catalyse the reversible formation of fructose 1,6 bisphosphate from dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde 3-phosphate (G3P) (Sprenger *et al.*, 1997). This enzyme exhibits a broad pH spectrum and unusually high temperature stability (Gustavo, 2000) meaning that it can be utilised to produce chiral building blocks in chemoenzymatic synthesis (Sprenger *et al.*, 1997).

Transketolase interactions have been studied using the isolated enzyme and most of the reported literature has focused on the biocatalyst in isolated form. Identifying transketolase properties in intracellular environments is a complex task due to the intricate metabolic pathways it is naturally involved in. The solution has always been to remove transketolase from its natural network and apply it to simpler reactions so that research can be carried out more effectively. The model reactions used have been mostly irreversible ones, which have allowed easy detection of the kinetics and other distinguishing properties of the enzyme (Figure 1.12). In recent studies a prominent model reaction used to study transketolase is one in which the enzyme forms a new carbon-carbon bond between glycolaldehyde and hydroxypyruvate. Although not the natural mechanism of transketolase the reaction is irreversible and it has also been applied to measuring transketolase activity (Figure 1.12).

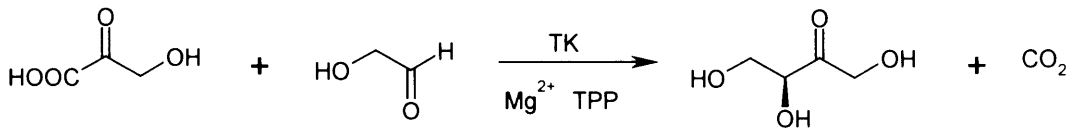


Figure 1.12 The production of L-erythrulose using transketolase from HPA and glycolaldehyde.

Hydroxypyruvate is used as a ketol donor. The reaction is irreversible because carbon dioxide is produced which is not an acceptor aldehyde.

1.2.6 Model reaction system

Incorporating the action of FruA, TPI and TK is the production of X5P used as the model reaction system in this thesis. It is one of the few cases where other aspects of the transketolase action have been focused on. This reaction scheme is attractive as it is also irreversible at the same time showing transketolase working with other enzymes in the presence of a variety or substrates. The synthesis of D-xylulose 5-phosphate from Fru1,6BP is illustrated in Figure 1.14. The multi-enzymatic process can also be broken down to start with DHAP as a substrate as shown in Figure 1.15. Other in-depth studies on the preparation of X5P have been highlighted in Section 1.3.

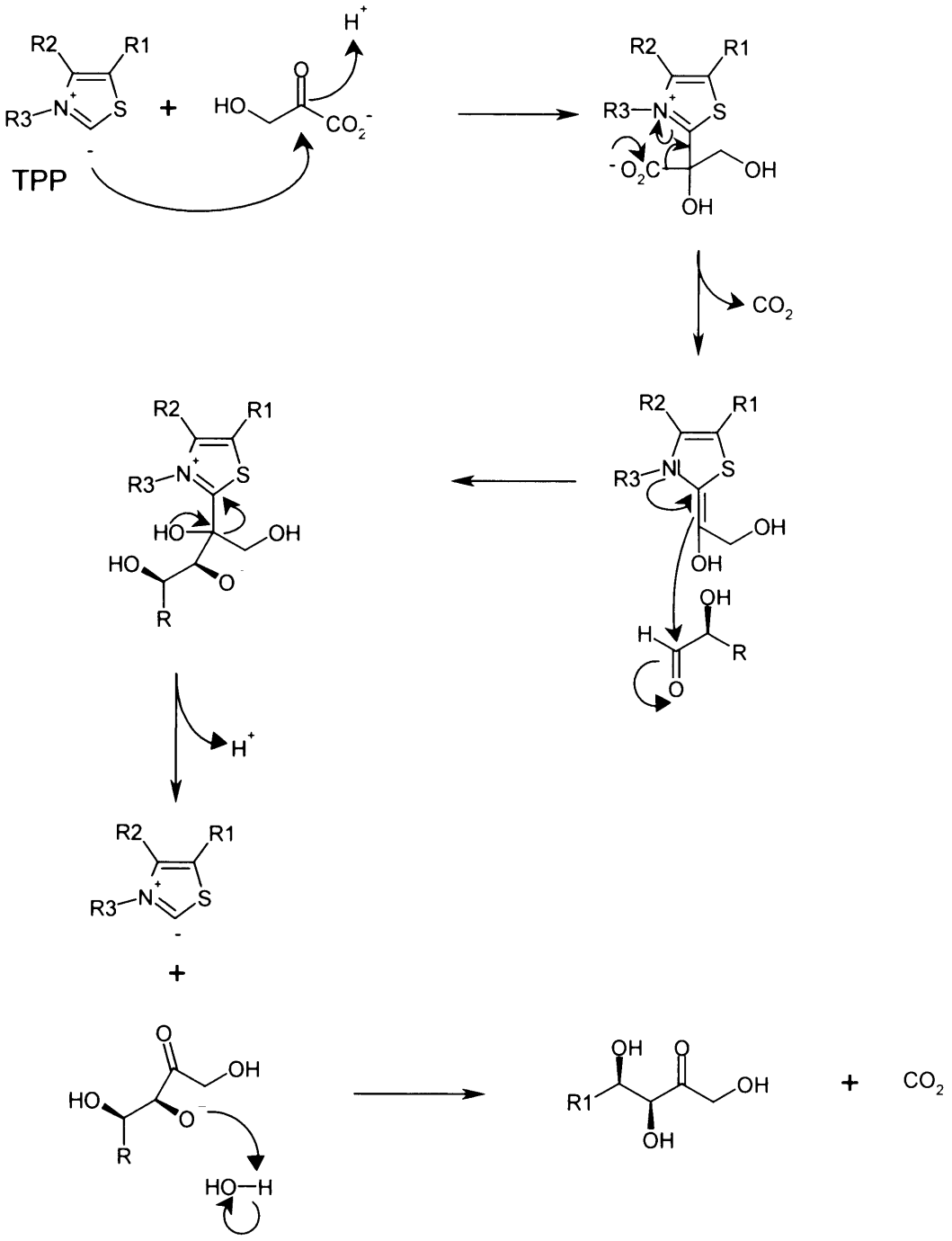


Figure 1.13 Mechanism of action of TPP in transketolase catalysed carbon-carbon bond formation (Booth and Nixon, 1993).

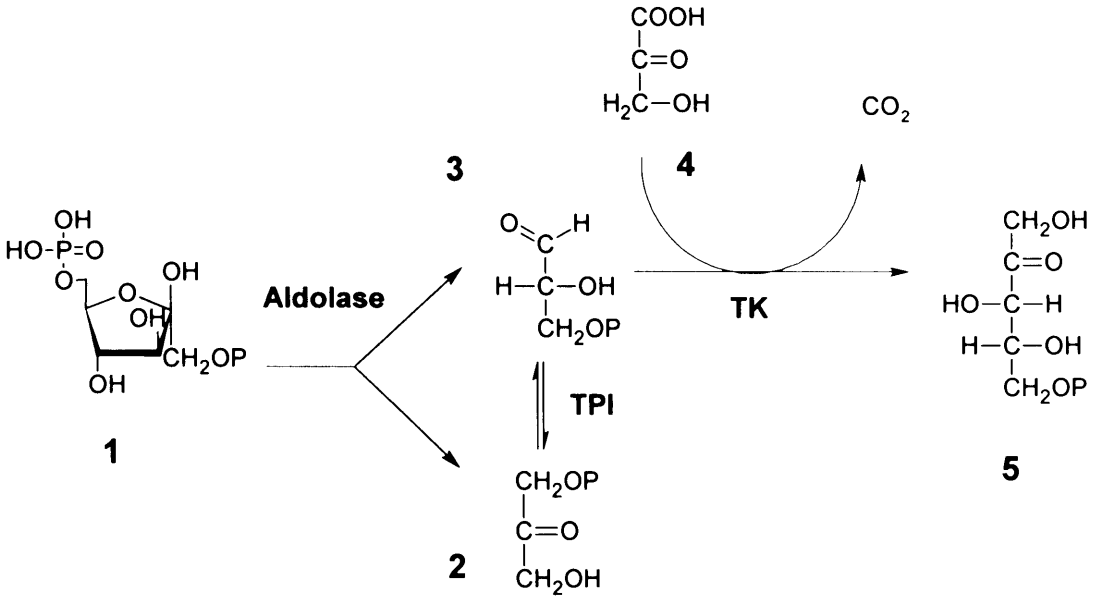


Figure 1.14 The enzymatic preparation of X5P (5). Aldolase breaks Fru 1,6 BP (1) to produce DHAP (2) and G3P (3). Meanwhile DHAP is converted to G3P by TPI. TK subsequently converts G3P to X5P using HPA (4) as a ketol donor. (based on findings from Zimmermann *et al.*, 1999).

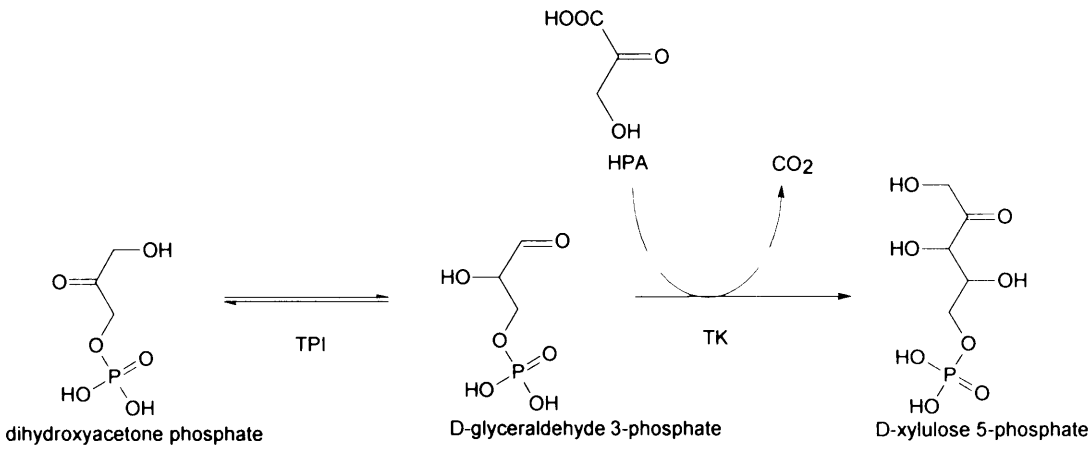


Figure 1.15 The production of D-xylulose 5-phosphate (X5P) from dihydroxyacetone phosphate (DHAP) involving D-glyceraldehyde 3-phospahte (G3P), triosephosphate isomerase (TPI) and transketolase (TK)

1.3 The multi-enzymatic production of X5P

The enzymatic synthesis of xylulose-5-phosphate was described in 1957 (Horecker *et al.*, 1957) leading to the analysis of the various systems involving X5P. The role of stereoisomerases in arabinose fermentations was established where the conversion or ribulose-5-phosphate to xylulose-5-phosphate was analysed (Wolin *et al.*, 1957). Some of the early systems studied are listed in Table 1.1.

Reaction	**Biocatalyst involved**	**Reference**
D-xylulose 5 phosphate ⇆ ribulose 5-phosphate	D-xylulose5-phosphate 3-epimerase	(Racker, 1962).
D-Xylulose 5-phosphate + Pi → Acetyl phosphate + G3P	D-xylulose 5-phosphate phosphoketo lase	(Horecker, 1962)
L-Ribulose 5-phosphate ⇆ L-Xylulose 5-phosphate	L-Ribulose 5-phosphate-4-epimerase	(Horecker, 1962)
D-Xylulose 5-Phosphate ⇆ D-Ribulose 5-Phosphate	D-Xylulose 5-Phosphate-3-epimerase	(Hurwitz, 1962)
D-Xylulose + ATP → D-Xylulose-5-phosphate + ADP	D-Xylulokinase from calf liver	(Ashwell, 1962)

Table 1.1 The reactions studied involving X5P.

The works represented in Table 1.1 provided the basis for the preparation and analysis of mixtures of D-ribose 5-phosphate, D-ribulose 5-phosphate, and D-xylulose 5-phosphate (Wood, 1975). For assay purposes an enzymic method was a developed for the determination of fructose 6-phosphate, xylulose 5-phosphate, and sedoheptulose 7-phosphate (Kochetov, 1982) aiding preparative enzymic synthesis and isolation of D-threo-2-pentulose 5-phosphate (D-xylulose 5-phosphate) (Mocali *et al.*, 1985) followed by synthesis of 1-Deoxy-D-xylulose-5-phosphate (Thiel and

Adam 1999) together with enzymatic synthesis of sedoheptulose 7-phosphate and ido-heptulose 7-phosphate (Sungsook *et al.*, 1999).

The most recent study of the preparation of X5P has been conducted by Fessner and co-workers (Zimmermann *et al.*, 1999). It has been highlighted that X5P has been implicated to serve as a second messenger in liver tissue for the stimulation of glycolysis by activating a regulatory protein phosphatase (PP2A). It has also been found to play an important role in Vitamin B6 biosynthesis (Laber *et al.*, 1999).

1.3.1 Kinetics and thermodynamics

The study of kinetics and thermodynamics of a given biocatalytic system is important in identifying its traits for subsequent process development (Kolchetov, 2004). Transketolase reactions have been studied in terms of their kinetics and thermodynamics to make comparisons of different sources of the biocatalyst (Masri *et al.*, 1988) and the kinetics of overexpressed transketolase has now been illustrated in literature. Gyamerah and Willetts (1997) provide one example of this. The Michaelis and inhibition constants were determined. However as mentioned before the Michaelis-Menten kinetics are used to describe one-substrate systems and therefore not directly applicable to the model system chosen for this work. Double-reciprocal plots for inhibitory levels of HPA, erythrulose and glycolaldehyde were drawn and the initial rates and inhibition data were obtained. Where the efficient use of an enzyme is critical, in-situ product recovery (ISPR) has been suggested (Chauhan *et al.*, 1997) in presence of a really toxic product/substrate. Operating in a fed-batch mode may negate the need for ISPR. Immobilisation has been shown to have a significant effect on stabilising the enzyme activity. This improvement in stability must however be traded off for the activity (Brocklebank *et al.*, 1998). Kinetics of multi-enzymatic systems have been described based on initial rates (Purich, 1995) and yield based on biocatalyst (Biebrich and Yu, 1999). More recently with the aid of computers kinetics has been established using a progressive curve fitting (Straathof, 2001) and modelling of reaction kinetics has been used for reactor selection (Vasic-Racki *et al.*, 2003).

When considering the thermodynamics many biocatalytic conversions involve reactions with an unfavourable equilibrium (Humphrey *et al.*, 2000). The law of mass action will apply such that the removal of one species from the reaction

mixture will shift the equilibrium position. This is where the level of substrate can effect biocatalytic conversions. This equilibrium links directly to the yield, which is preferred to be as high as possible. In this work thermodynamics refers directly to the equilibrium and therefore the resulting yield of product on substrate. The thermodynamics of a biocatalytic conversion is often referred to as the return of product from substrate directly influenced by substrate concentration resulting in changes in yield (Wolff *et al.*, 1999).

1.3.2 Assays for transketolase activity

Several assays have been developed for measuring transketolase activity. Henrich and co workers illustrated a method for this using a series of enzymes (Henrich *et al.*, 1972), (Hobbs *et al.*, 1996). This sequential enzymatic assay has been used and modified but the basis remains the same. The multi-enzymatic assay for transketolase activity is illustrated in Figure 1.16. The major limitation of using this assay has been the dependency of the transketolase reaction on the rest of the enzymes. Transketolase is limited by the activity and the presence of the other enzymes and generally the assay is difficult to perform with great accuracy. This highlights the need to study the behaviour of transketolase when involved in a multi-enzymatic system.

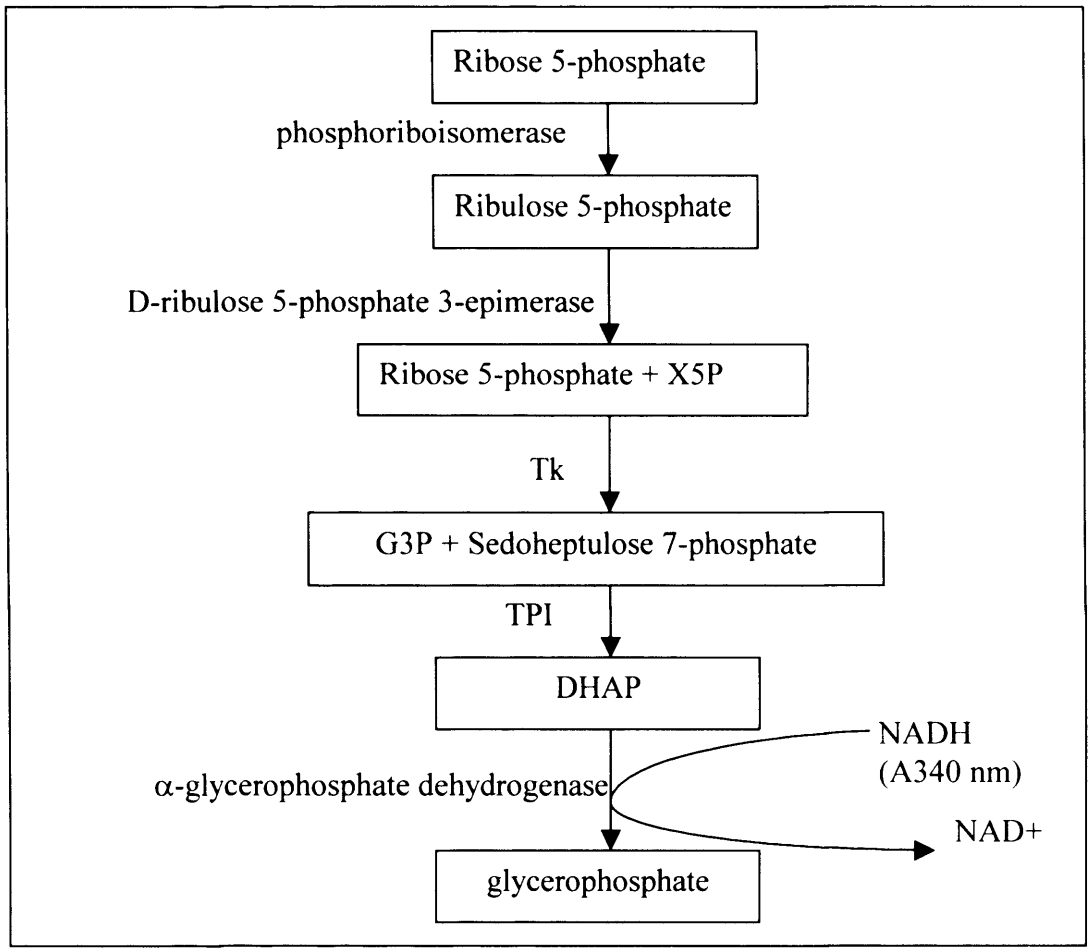


Figure 1.16 The enzyme linked assay system developed for analysing transketolase activity (Hobbs *et al.*, 1996). TK source (0.001-0.03 U mL^{-1}) is added to a mixture containing TPP, $MgCL_2.6H_2O$, NADH, α-glycerophosphate dehydrogenase, TPI, Phosphoriboisomerase, D-ribulose 5-phosphate 3-epimerase and lastly Ribose 5-phosphate in glycyglycine buffer, pH 7.6. and 37 °C.

More recent activity assays have been developed by applying irreversible reactions with transketolase so that rates of production can be accurately measured. One of these model reactions is the L-erythrulose production as shown previously (Figure 1.12).

Bolte and co workers (1998) developed an assay in the laboratory, which uses L-erythrulose as a ketose donor which results in the release of glycolaldehyde, which is reduced by NADH in the presence of yeast alcohol dehydrogenase, allowing UV spectroscopic monitoring (Andre *et al.*, 1998). Mitra and Woodley (1996) developed an accurate method for the direct quantitative analysis of HPA,

glycolaldehyde and L-erythrulose using HPLC. This meant that transketolase catalysed biotransformations of HPA with water-immiscible aldehydes could be monitored by the measurement of the loss of HPA. This is a more direct assay for transketolase, monitoring the components in a biotransformation using HPLC (Gyamerah and Willetts, 1997).

1.3.3 Information on reactants and reaction conditions (Component characterisation)

The *E. coli* transketolase catalysed carbon-carbon bond formation has been used in developing methods of biotransformation characterisation for reactor evaluation and selection. Here Mitra and co workers described a set of experiments needed to determine constraints and limits of a biotransformation in a reactor (Mitra *et al.*, 1998).

These key experiments were:

- Measurements of the solubility of substrates. Where for HPA large differences in solubility were observed between the free acid and the lithium salt in the pH range of 2.0-8.0. Generally the solubility decreased with increasing pH.
- Effects of pH on substrates and product. Here HPA, glycolaldehyde and erythrulose were shown to be sensitive to alkaline environments. The rate of degradation was concentration dependent.
- Effects of pH on transketolase. The stability of holotransketolase is maintained between pH 5.5 and 10.0 and the optimal range for activity is 7.0-7.5. It must be noted that Sprenger *et al.*, 1995 showed purified transketolase to be optimal at pH 8.5, crude extracts have been used successfully in reactions in the lower pH range. This enzyme is denatured irreversibly at all pH values less than 6.5.
- Effects of reaction components on transketolase.

500 mM HPA also causes this level of activity loss after only 6 hours. Based on the above information the constraints and relevant process parameters for a biocatalytic system were described. It was hence pointed out by Mitra and co workers that investigations are required when characterising any transketolase system based on four key areas:

1. Alkaline stability of aldehyde and product.
2. Solubility of aldehyde and the effect of temperature and pH on solubility.
3. Stability of the holotransketolase in the presence of aldehyde substrate and the effects of aldehyde concentration.
4. Potential for product and substrate inhibition.

These investigations highlight the need to gather information on the reactants and biocatalysts forming the basis for characterisation of a model synthetic reaction in order to define the constraints for reactor selection and scaleup (Mitra *et al.*, 1998).

1.3.4 Development of large-scale biotransformations

There are various parameters that are critical for developing a large-scale process. In those involving transketolase as a key step it has been noted that the starting aldehyde, glycolaldehyde causes deactivation of the enzyme thereby placing a limit on the concentration of the starting material that can be used. Inhibition has also been investigated and potential solutions via in situ product removal (ISPR) reported. After considering a number of possible methods including ion-exchange, complex formation, physical absorption, it has been found that the use of phenylboronic acid to specifically form the phenylboronate derivative of the vis-diol products such as erythrulose offered the most promise. By using an immobilised phenylboronate resin (Affigel 601) it has been possible to remove erythrulose together with some glycolaldehyde (21% on a molar basis) from a mixture containing these two components and HPA which did not bind. At large scale a productivity level as measured by space-time yield (STY) of 1.9 g $L^{-1}hr^{-1}$has been reported in terms of erythrulose. STY indicates the concentration of product (g L^{-1}) in the reactor at the end of a process divided by the total reaction time (hr) and can also be used as a term to describe the productivity and efficiency of that process (Chauhan *et al.*, 1997). The affects of immobilising *E. coli* Tk on two commercially available supports (Eupergit C and Amberlite XAD-7) has been reported with improvements of 80-100 fold in stability (Brocklebank *et al.*, 1999).

1.3.5 Operating maps / windows

As part of a structured approach to biotransformation process selection and design, windows of operation have been put forward as a design tool enabling the selection of the correct process based on a fundamental characterisation of the reactants, product reaction and biocatalyst. These operating maps / windows were developed by Woodley and Titchener-Hooker (1996) to plot boundaries, determine the optimal operating points and feasible regions of operation for a given system. As a design tool windows of operation have been applied to a variety of systems in the literature including the characterisation of the chemoenzymatic synthesis of N-acetyl-D-neuraminic acid (NEU5AC) where substrate concentrations were used as process limits to determine areas of insufficient operation (Blayer *et al.*, 1996). They have also been applied to define reactor operation giving guidance for feeding and pH control (Woodley *et al.*, 1996) and have defined economic scenarios and process options for two enzyme reactions in a linked cofactor recycle system for chiral lactone synthesis (Hogan and Woodley, 1999). More recently visualising bioprocesses using a 3D windows of operation has been suggested providing a more detailed analysis and the confidence to select the correct process (King *et al.*, 2004).

1.4 Biochemical process engineering aspects

Chemical processes have long benefited from engineering problem analysis. This has brought about a high level of understanding in terms of process variables, material balances and energy balances. This is not the case with biochemical processes mainly due to their relatively short existence in industry. It is important to apply chemical engineering aspects and evolve them for biocatalytic systems. Firstly some key chemical engineering principles must be taken into consideration.

1.4.1 Bioprocess development

Rudd and co workers in the year of 1973 pulled together examples of process selection and drew some clear guidelines emphasising the history of processing and outlining a way of thinking useful in discovering technology for process problem solving (Rudd *et al.*, 1973). At the heart of this study were two broad kinds of

activity, synthesis and analysis important during the development of a new and useful process. Chemical processes have been developed using these guidelines on process synthesis and conceptual design (Douglas, 1998). To overcome the complexity of large engineering tasks they are decomposed so that each level is understood and defined. Based on a hierarchical approach a design methodology is defined. Sets of possible processes are identified using the design methods. The process designer determines if a process will be profitable based on a set of flowsheets. This conceptual design as long been implemented as a powerful tool for the synthesis of chemical processes (Han *et al.*, 1995). These techniques are still being further refined in the chemical industry where minimization of the time-to-market of products is really becoming a key issue in process development (Schultz *et al.*, 2000).

Biocatalysis development often focuses on the maximum yield achievable by a process rather than whether it is profitable. Profitability must be addressed very early in bioprocess development as more multi-step reactions are being proposed (Straathof *et al.*, 2002). In the chemical industry screening reaction pathways and calculating stream costs are common practice. The multi-enzymatic production of xylulose 5-phosphate is an example of a complex system with a variety of elements and many intricate relations within it. Systems such as this one are notoriously difficult to implement in industry. Understanding the multi-enzymatic complex is very important in particular properties of all the substrates and enzymes.

Multi-criteria process synthesis has been applied to a variety of systems such as that of a penicillin manufacturing process where it has enabled the identification of a set of flowsheets that were sustainable economically and environmentally. The best flowsheets or processes are chosen based on a set of criteria (Steffens *et al.*, 1999).

Using a kind of analytical system will drastically reduce the biochemical process development time and enable the biocatalytic process to compete with the chemical industry. This type of analysis has not yet been completely applied to a biocatalytic system where is absolutely essential in eliminating unattractive processes early on in development. In particular as procedures efficient and useful in the laboratory can be unattractive at commercial scale (Straathof *et al.*, 2002).

At commercial scale, high product concentrations are necessary if a process is to be considered viable and typical industrial yields are expected to be in the magnitude of grams. These requirements will ensure profitability after downstream processing

and possible losses. Process development is synthesizing processes that are very quantitatively identical to current technology where predictions are possible at an early stage.

Today's biocatalysis carries with it many tools. These must all be proposed together in a process development paradigm for future success of biocatalytic systems. This means that analysis and evaluation of process and biocatalyst options leads to modifications that later bring about synthesis of more economic process models (Lye *et al.*, 2002).

The study must start with the gathering of information with regards to the combination of often-diverse concepts involved in the multi-enzymatic preparation. This in turn enables the examination of the synthetic complex, its elements and their relation to each other at the stage of process assessment. Process assessment as it is termed will open up opportunities as to what are the best synthetic options for industrial use. This is similar to shortcut methods that have been devised in chemistry to eliminate poor choices and ranking attractive alternatives (Mahajanam *et al.*, 2001).

When solving the multi-enzymatic system it is important to decompose the problem into several easier bite-sized ones, each being the individual enzymatic steps. By considering the auxiliary operations involved it is possible to devise a set of complete process flowsheets. Once this is carried out the options for different processes are evaluated using a scoring system. The sensitivity of this system is to be evaluated thoroughly before it is deemed to be a valuable and comprehensive model (Schultz *et al.*, 2000). A Hierarchical process development strategy for use in biocatalysis can be proposed here adapted from those used in chemical engineering (Figure 1.17).

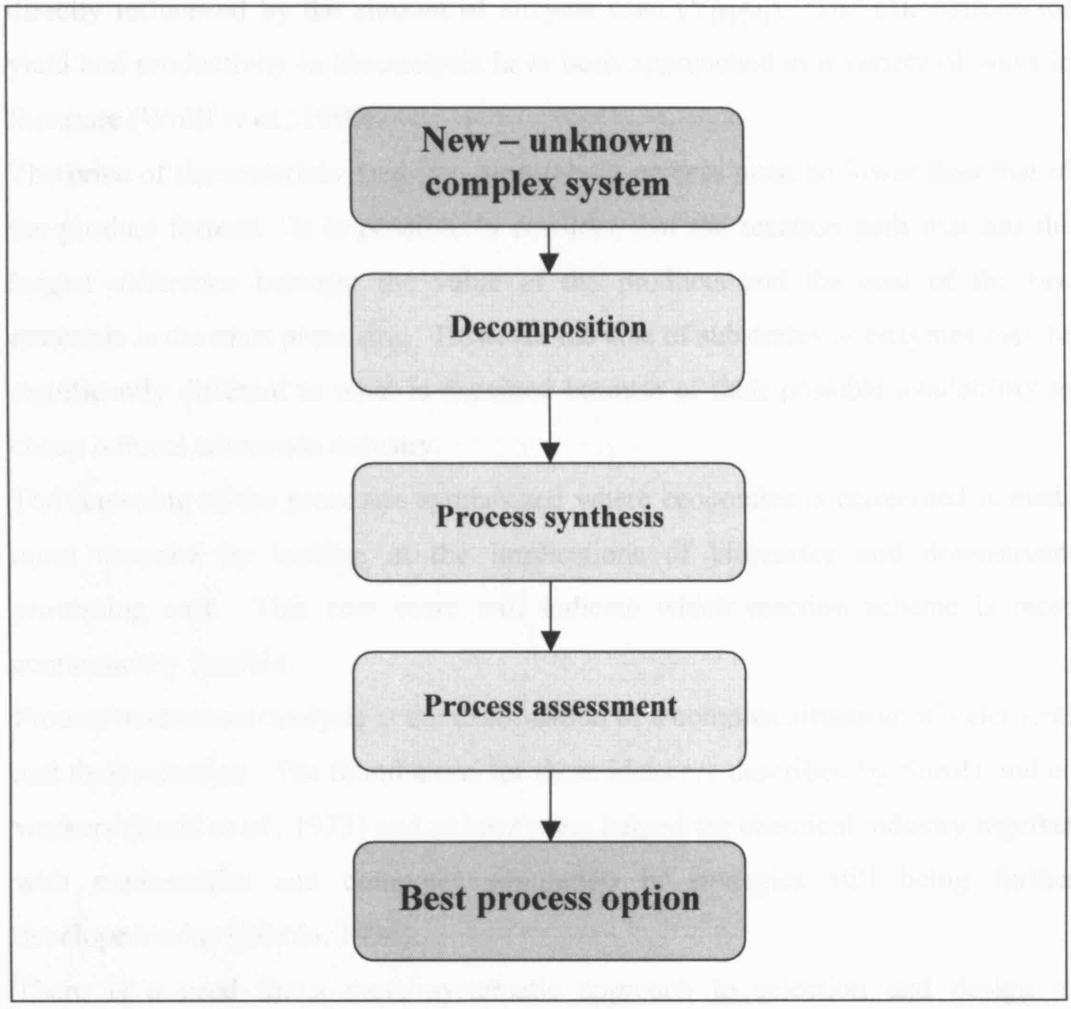


Figure 1.17 A process development strategy in biocatalysis adapted from those used in chemical engineering.

Model based bioprocess optimization has received a lot of attention in recent years. However it is clear that mathematical modeling in optimization of bioprocesses by itself has many limitations and needs to be accompanied by real scientific knowledge at every step when dealing with the dynamics of bioprocesses (Van Impe *et al.*, 1996).

An efficient process is addressed in terms of yield, product concentration and volumetric productivity. Nearly a quarter of industrial biotransformation processes operate to produce carbohydrates and other components such as xylulose 5-phosphate. As in the chemical industry these product developments must give a

high yield of approximately 80% (Straathof *et al.*, 2002). In biocatalysis this yield is based on the amount of product achieved from substrate ($Y_{[P]/[S]}$). Kinetics are directly influenced by the amount of enzyme used ($Y_{[P]/[E]}$). The calculations for yield and productivity in biocatalysis have been approached in a variety of ways in literature (Wolff *et al.*, 1999).

The price of the materials used in a biocatalytic process must be lower than that of the product formed. It is possible to consider that the reaction path that has the largest difference between the value of the products and the cost of the raw materials is the most promising. However the cost of substrates or enzymes may be significantly different to what is expected because of their possible availability as cheap internal sources in industry.

The screening of the processes synthesized where economics is concerned is made more accurate by looking at the implications of bioreactor and downstream processing cost. This cost score will indicate which reaction scheme is more economically feasible.

Process assessment/analysis is the examination of a complex situation; it's elements and their relations. The foundations for these ideas are described by Siirola and co workers (Rudd *et al.*, 1973) and in later years helped the chemical industry together with mathematics and computer simulation in strategies still being further developed today (Siirola, 1996).

There is a need for a more systematic approach to selection and design of biotransformation processes. In many cases process engineers can benefit from their synthetic potential and reduce plant waste by cutting down on process steps.

It is however very difficult to develop a scalable process from laboratory scale reactions. The success of scaling up a biocatalytic process for example relies hugely on a set of decisions made early on in process design.

1.4.2 Process synthesis

Process synthesis is the combining of diverse concepts into a coherent whole. Most products are several reaction steps away from readily available raw materials. Invariably there are several possible reaction paths to a product. The only completely accurate way to assess the commercial attractiveness of a reaction path

is to synthesise a whole set of possible routes. This will form the basis of then analysing each to find the most attractive option.

Synthesis is creative action taken based on what is known on a particular scientific system where a series of process options are composed and constructed.

At this stage all the knowledge known about a system must be put to good use. Applying this to biocatalysis this points to the attributes of the biocatalyst, substrates and other reaction components. Armed with this scientific data initially gathered in the laboratory it is possible to conceptualise a variety of possible processes for production.

Further more on reaction path analysis there is an internal dialogue between the chemist/biochemist and the engineer. Meaning that a set of questions and answers enable this creative process. The science is put forward as fact and engineering takes place based on these facts. A set of key questions can aid process synthesis:

1- What are the main reactions and major side reactions?
2- In what phase do the reactions take place and what are the phases of the pure reactants and pure products?
3- What are the environmental features such as temperature and pressure of the reaction?
4- What are the catalysts properties?
5- What solvents are used?
6- What is the quality of the reactants used?
7- What yields were obtained of the product and the by-products?
8- What are the details of the laboratory procedures used during reaction and product purification?

By examining the scientific answers to these basic questions the engineer can synthesise or come back with a set of possible solutions or processes. This is the marriage of scientific research and engineering design. The questions raised during process synthesis will serve the important role of easing the transition from the laboratory to the commercial process. These questions are key and are all equally important depending on the biochemical reaction at hand. The answers to the questions raised are either experimentally derived or estimated based on logic and

can form a set of input parameters for a decisional tool to synthesise a set of possible process options (Figure 1.18).

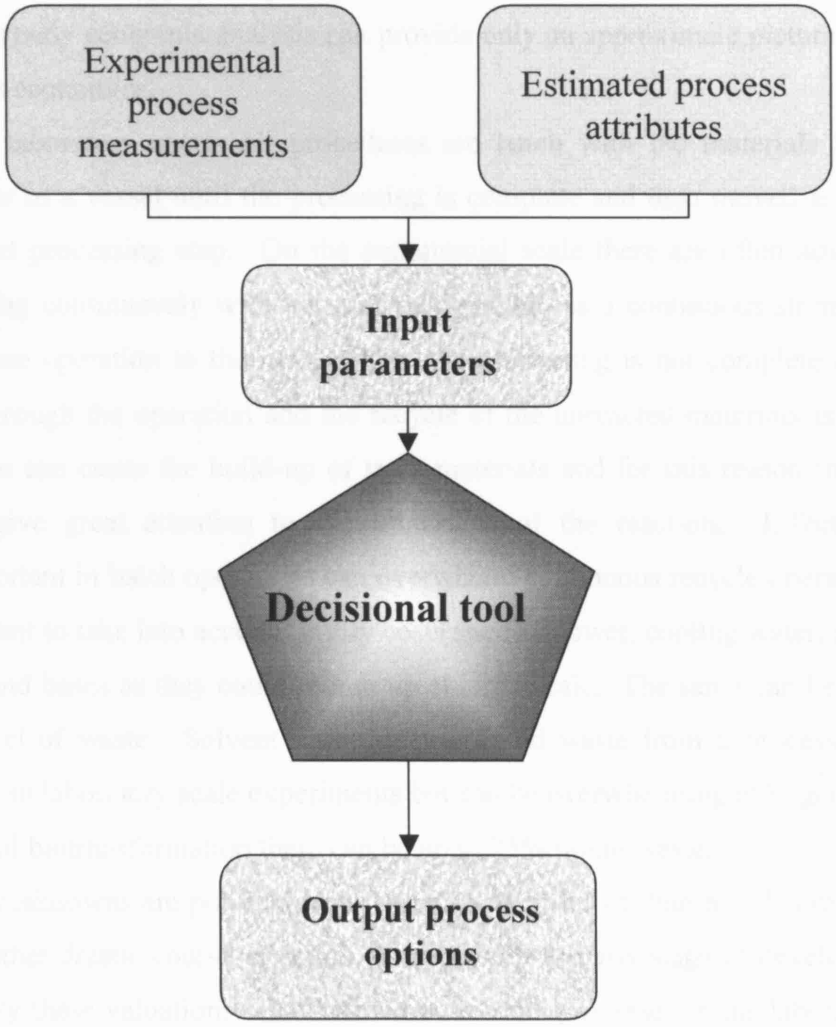


Figure 1.18 A possible decisional tool for rapid process synthesis.

1.4.3 Process assessment

All things being equal the reaction path that has the largest difference between the value of the products and the cost of the raw materials is the most promising (Rudd *et al.*, 1973). A screening criterion is useful to eliminate uneconomic processes. Passing this test is however not sufficient to ensure economic viability. Many profitable processes exist that cannot be justified on published cost data because of for example internal cheap sources of otherwise expensive raw materials. For these reasons early economic analysis can provide only an approximate picture of the real process economics.

In the laboratory nearly all procedures are batch with the materials being kept together in a vessel until the processing is complete and then moved as a batch to the next processing step. On the commercial scale there are often advantages to operating continuously with the materials passing as a continuous stream in pipes from one operation to the next. Often the processing is not complete during one pass through the operation and the recycle of the unreacted materials is necessary. Recycle can cause the build-up of trace materials and for this reason the engineer must give great attention to the impurities of the reaction. Effects that are unimportant in batch operations can overwhelm continuous recycle operations. It is important to take into account utility costs such as power, cooling water, steam, gas, acids and bases as they could mount up at large scale. The same can be applied to the level of waste. Solvent waste or any liquid waste from a process can seem minute in laboratory scale experiments but can be overwhelming at larger scales. In a typical biotransformation there can be up to 75% liquid waste.

Cost breakdowns are possible using a variety of rules of thumb. This may seem to be a rather drastic course of action taken at such an early stage of development. In actuality these valuations when carried out on the processes in the laboratory serve to alert the researcher of industrially unfavourable systems. Typical estimated economics for a certain designed process takes into consideration the cost of the raw materials, the product and utility costs (Siirola, 1996). Having completely

analysed the economics of each process they can be compared to each other using three parameters for example conversion, raw material cost and profit.

Once the designs have been completed it is important to analyse them in detail. Flowsheets must be drawn up with regards to the attributes of each process synthesised. The information on each process will distinguish each one from the rest. Once all this information is gathered it is possible to differentiate between processes and identify the better options or more importantly eliminate the unattractive ones early.

1.5 Multi-enzymatic process development

Based on the research the current approach to bioprocess development is research a given process, test it in the laboratory, optimise it and develop it for larger scale. A new paradigm is possible in the form of process synthesis based on key experiments followed by process assessment leading to a reliable and scalable process.

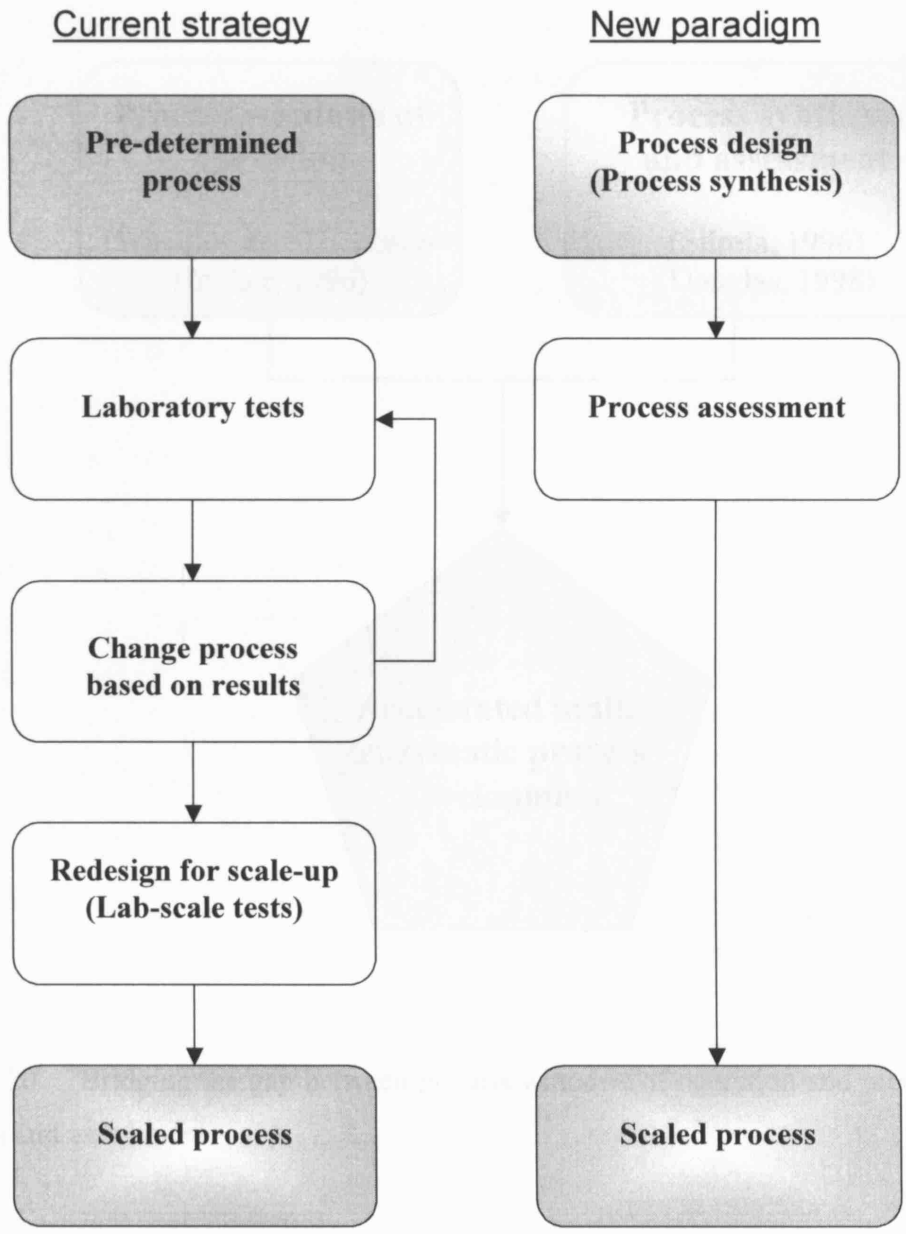


Figure 1.19 A new paradigm for rapid bioprocess development.

Achieving the new paradigm suggested in Figure 1.19 is possible by drawing from two schools of thought in process assessment. By bridging together the characterisation studies, windows of operation (Woodley and Titchener-Hooker, 1996) and process synthesis (Siirola, 1996 and Douglas, 1998) it is possible to make advancements in the field of bioprocess development (Figure 1.20). This will in turn provide a better understanding of multi-enzymatic processes.

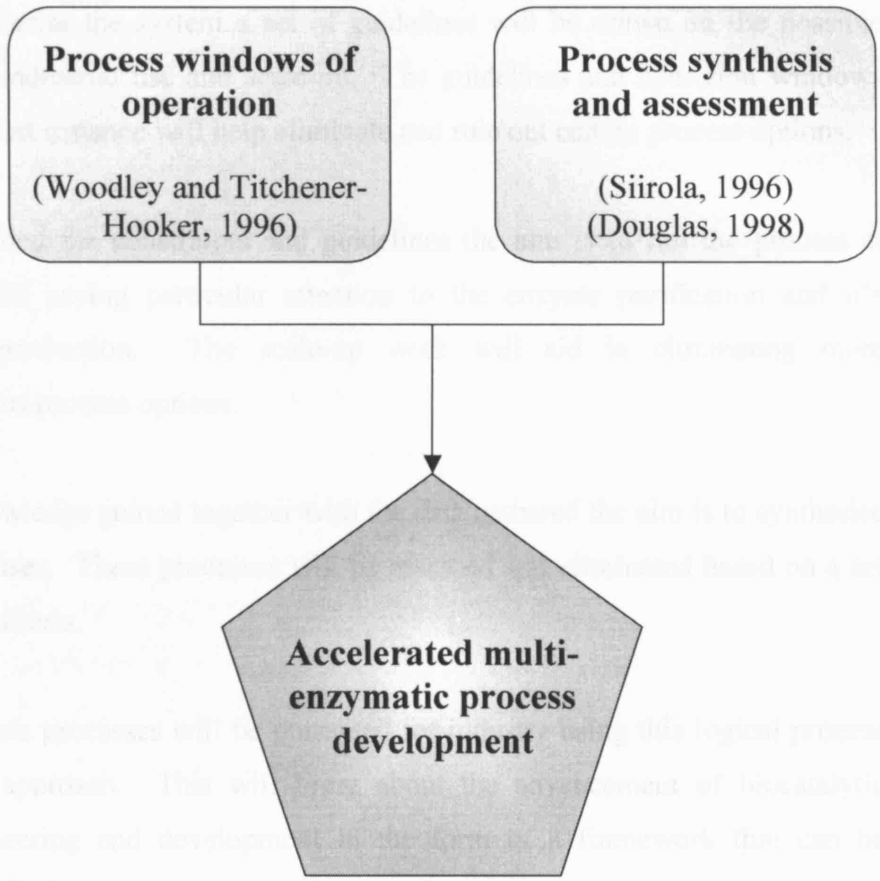


Figure 1.20 Bridging the gap between process windows of operation and process synthesis and assessment.

1.6 Aims of study

The aim of this thesis is to investigate the multi-enzymatic X5P preparation for process synthesis, assessment and selection.

The strategy is to first break down the complicated reaction scheme into small segments and characterise the key components gathering important data. Using the data to characterise the system a set of guidelines will be drawn on the possible processes for industrial use and scale-up. The guidelines and operation windows drawn in the first instance will help eliminate and rule out certain process options.

Having identified the constraints and guidelines the aim is to run the process at laboratory scale paying particular attention to the enzyme purification and it's impacts on production. The scale-up work will aid in eliminating more disadvantageous process options.

Using the knowledge gained together with the data gathered the aim is to synthesise a set of processes. These processes will be assessed and eliminated based on a set of important criteria.

A set of eligible processes will be presented for industry using this logical process development approach. This will bring about the advancement of biocatalytic process engineering and development in the form of a framework that can be eventually applied to more complicated and intricate enzymatic systems.

2 General materials and methods

2.1 Equipment and materials

2.1.1 Reagents and suppliers

All reagents used were of analytical grade and most were obtained from Sigma-Aldrich Co Ltd., (Poole, Dorset, UK) with the exceptions of DHAP, Fru1,6BP, and TPP, which were donated by Fluka (Buchs, Switzerland). FruA (rabbit crystalline suspension) was used with the activity of 20 U per mg of protein where 1 Unit converted 1.0 μmole of Fru1,6BP to DHAP and G3P per min at pH 7.0 at 25 °C. TPI (type II-S from rabbit muscle) was used with the activity of 5000 Units per mg of protein. 1 Unit converted 1.0 μmole of G3P to DHAP per min at pH 7.0 (Sigma-Aldrich, Poole, Dorset, UK). Transketolase (*E. coli*) obtained from Sigma-Aldrich (Poole, Dorset, UK) was of 0.5 U mg^{-1} specific activity based on the conversion of 1 μmole of glycolaldehyde to erythrulose per min at pH 7.6. For the purification of xylulose 5-phosophate the AG strong anion exchange resin and AG 50W cation exchange resin were both obtained from Bio-Rad Laboratories Ltd. (Hemel Hempstead, Hertfordshire, UK). The Aminex HPX-87H, 300 mm x 7.8 mm chromatography column and the protein assay kit was also obtained from Bio-Rad laboratories Ltd., (Hemel Hempstead, Hertfordshire, UK). Reverse osmosis (RO) water was used in all experimental work.

2.1.2 Source of organism and maintenance

The strain used to obtain transketolase for the experiments was *E. coli* JM 107/ pQR 711 originally prepared by Dr. John M Ward at the Department of Biochemistry and Molecular Biology, UCL. This strain was used to prepare stock cultures, routinely made by suspending cells in glycerol solution (20% (v/v), 10 mL) and storing aliquots at -80 °C. Frozen stock cultures were reactivated by streaking onto fresh sterile agar plates.

2.1.3 Fermentation equipment

Fermentations were performed in 7L glass fermenter (LH fermentations Ltd., Maidenhead, UK) and a 75 L stainless steel fermenter (Inceltech LH SGI, Wokingham, UK). The exit gas was analysed and data reported by Propack™ fermentation logging software.

Parameter	**5 L fermentation**	**50 L fermentation**
Manufacturer	LH fermentation	Inceltech LH SGI
Total volume (L)	7	75
Working volume (L)	5	50
Sterilisation	*in situ*	*in situ*
Sparger	Ring	Ring
Baffles	4	4
Foam control	Chemical - Manual	Chemical - Automatic
Temperature control	Electrical	Jacketed vessel
pH control reservoirs	2	2
Impellers drive	Top	Top
Type	Turbine	Turbine
Number	3	3
Maximum air flow rate (L/min)	10	100

Table 2.1 Details of the fermenter vessels used

2.1.4 Chromatographic apparatus

2.1.4.1 HPLC equipment and reaction sample analysis

The reaction components were assayed for using a Dionex DX 500 chromatography system. The system comprised of a GP50 gradient pump, AS3500 auto sampler, LC30 chromatograph oven and ED40 electrochemical detector. Data collection and integration was performed using PeakNet™ software version 5.01. (Dionex Ltd., Camberley, Surrey, UK).

All reaction samples were diluted and later filtered using Whatman 25mm GD/X syringe filters (0.2μm PSU). The samples were then transferred into 2mL glass HPLC vials with inserts depending on the volume of the sample. Each sample was then injected into the system where it was passed through an Aminex HPLC organic acid analysis column (Aminex® HPX-87H Ion Exclusion Column 300mm x

7.8mm) at 60 °C together with a 0.1% Trifluoroacetic acid (TFA) solution as mobile phase. The injection volume was 1μL and the ED40 amperometry noise was set to 20 pC (integrated amperometry). The total analysis method time was a maximum of 15 minutes at 0.6 mL min^{-1} allowing ample time for all the reaction components to be eluted off the system. The retention times and typical reaction curves of the components are shown in Appendix I.

2.1.4.2 Reaction analysis using ED40 Electrochemical Detector

Conductivity and amperometry are specific and sensitive forms of electrochemical detection that have been combined in the ED40. These detection modes provide a complete method development tool. The triple pulsed amperometric detection allows detection of the compounds that require the working electrode surface to be continuously cleaned to prevent fouling. 500mL of 0.5 M NaOH solution was prepared freshly before each analytical period to be passed through the detector.

2.1.5 Monitoring of reaction vessels

All biotransformations and characterisation experiments were carried out in reaction vessels. Monitoring and control of the pH within reaction vessels was carried out using a VIT90 video titrator (Radiometer Ltd. Copenhagen, Denmark) comprising of an auto titrator unit and a stirred reaction vessel collectively referred to in this thesis as the pH stat. The reactions were monitored based on pH and acid consumption (HCl 0.5 M). The pH stat method ensured that the pH did not drift due to carbon dioxide release during the reaction. Figure 2.1 is an image of the pH stat used in all the experiments to control pH.

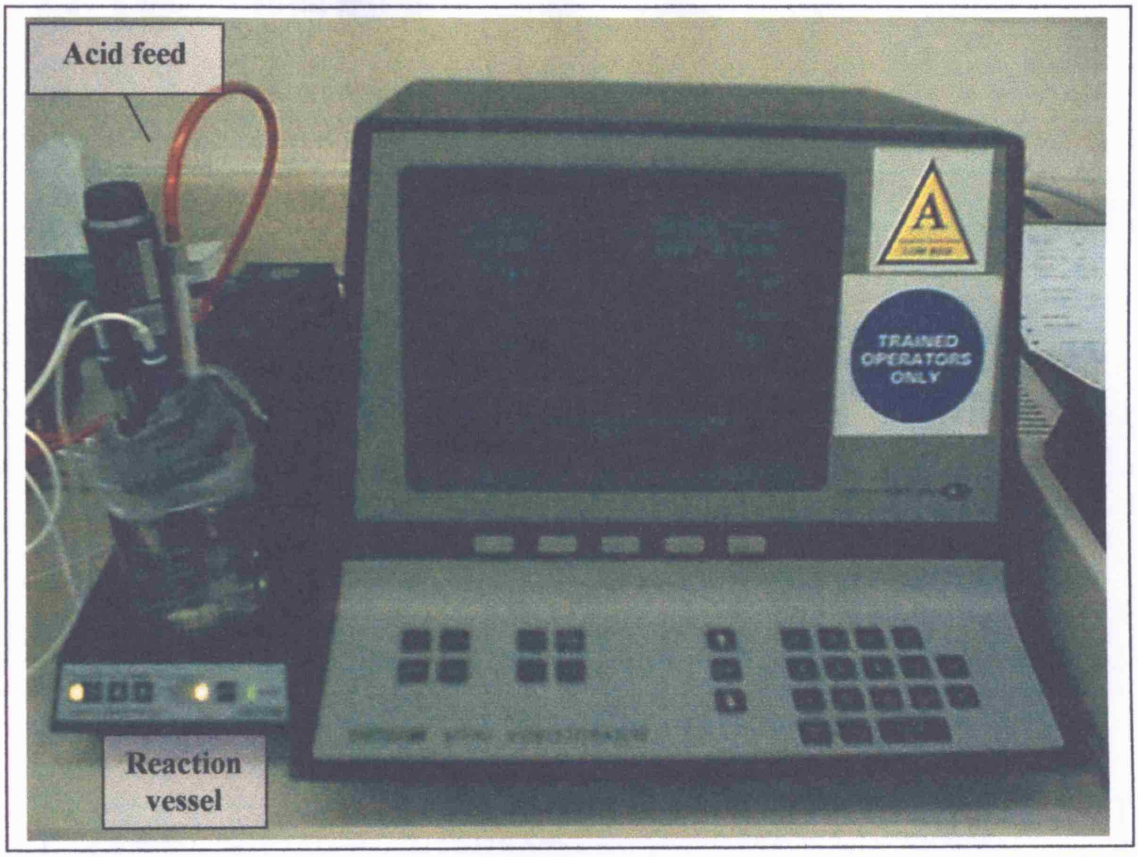


Figure 2.1 The monitoring of reactions using a pH-stat and a stirred baffled reaction vessel.

2.2 Experimental methods

2.2.1 Culture media for the production of transketolase

For the purposes of obtaining transketolase *E. coli* JM107/pOR 711 fermentations were conducted. These were carried out in particular media compositions based on the stage and the type of fermentation. The following diagram explains the different stages of *E. coli* JM 107 / pQR 711 cultivation.

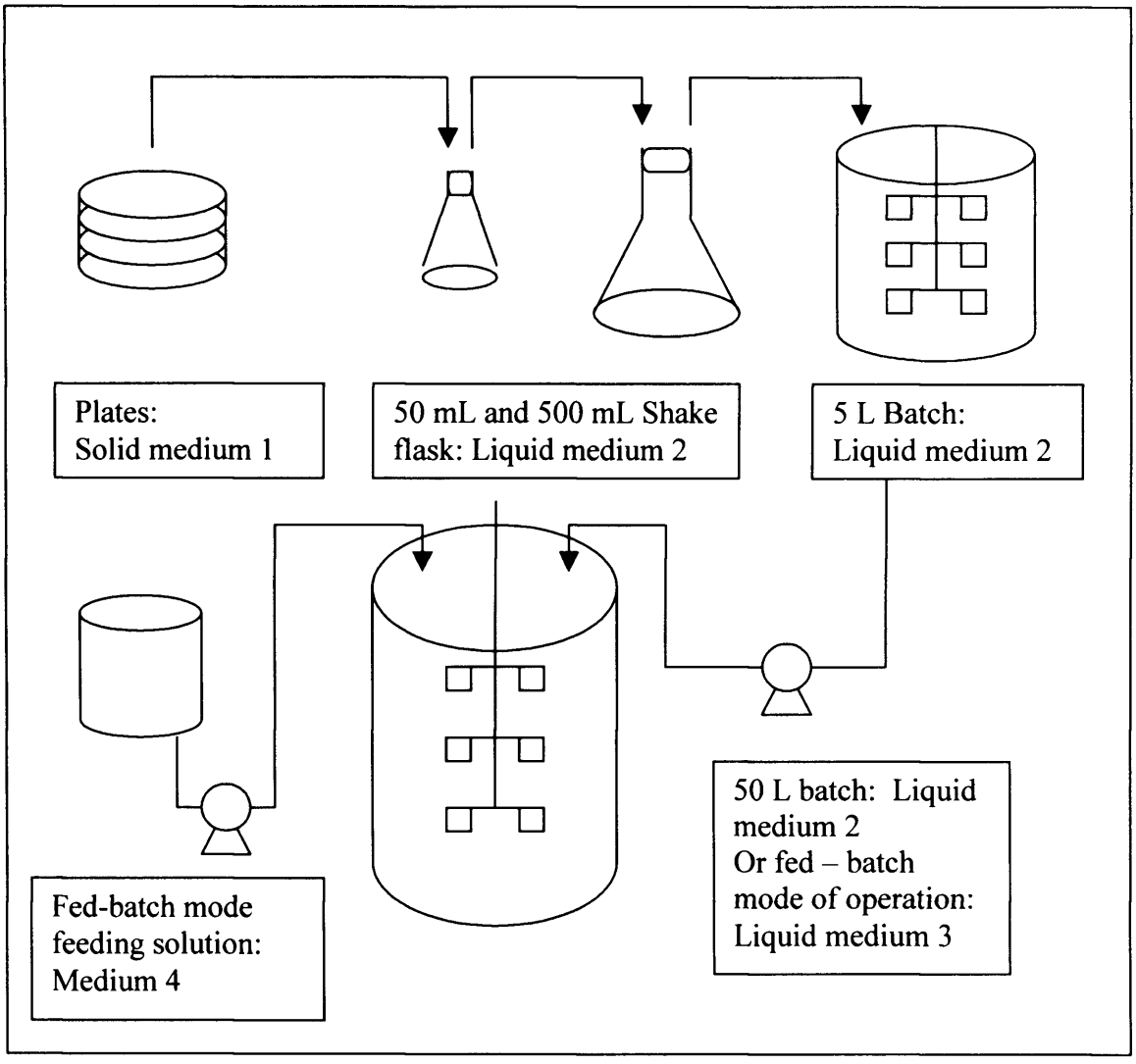


Figure 2.2 The various stages of *E. coli* JM 107/pQR 711 fermentations indicating the type of media and vessels used at each step.

2.2.1.1 Solid culture media

The components used for growing *E. coli* on agar plates before transfer into shake flasks are shown in Table 2.1. All the components were added to RO water except ampicillin. The media was autoclaved (121 °C for 30 minutes before cooling). Once the media had cooled to 50 °C the ampicillin was added by filter sterilisation via a 0.2 μm filter (Pall, MI, USA). The media was divided into plates and allowed to set prior to inoculation.

Component	**g/L**	**Sterilisation**
Glycerol	1	Autoclave
Tryptone	10	Autoclave
Yeast Extract	5	Autoclave
NaCl	5	Autoclave
Technical Agar	15	Autoclave
Ampicillin	0.5	Filtration

Table 2.2 The composition for the solid cultures (medium1).

2.2.1.2 Liquid culture media

In the preparation of the media it was found that the composition of the media suggested in the literature often resulted in precipitation. To avoid this problem the components were divided into 5 groups (A-E). The primary aim was to separate the phosphates from the sulphates during sterilisation. Groups A, B, C and D were prepared separately. The glycerol and magnesium sulphate (group A) were sterilised in the bioreactor. Groups B, C and D were autoclaved and added to the bioreactor before the start of the fermentation. The thiamine and ampicillin (Group E) were the final components to be added. These components were added before inoculation and were filter sterilised due to their sensitivity (Hobbs *et al.*, 1996).

Component	Medium 2 (Batch mode) Concentration (g/L)	Medium 3 (Fed-batch mode) Concentration (g/L)	Sterilisation group
Glycerol	50	5	A
$MgSO_4.7H_2O$	0.66	0.66	A
K_2SO_4	6.62	6.62	B
NH_4Cl	1.32	1.32	B
Na_2HPO_4	2.1	2.1	B
KH_2PO_4	3.96	3.96	B
$(NH_4)_2SO_4$	2.5	2.5	B
$MnSO_4.4H_2O$	0.00057	0.00057	C
$CuSO_4.5H_2O$	0.00033	0.00033	C
$ZnSO_4.7H_2O$	0.0032	0.0032	C
$CaCl_2$	0.33	0.33	C
Citric acid	0.66	0.66	D
$Fe_2(NH_4)2(SO_4)_2$	0.60	0.60	D
Thiamine	0.007	0.007	E
Ampicillin	0.125	0.125	E

Table 2.3 The composition of growth media 2 and 3 showing the components in different sterilisation groups to avoid precipitation.

Component	Concentration (g/L)	Sterilisation
Glycerol	200	Autoclave
$MgSO_4\,7H_2O$	8.25	Autoclave
Ampicillin	0.125	Filter sterilised

Table 2.4 Fermentation of *E. coli* feed composition (medium 4).

2.2.2 Transketolase production

2.2.2.1 Shake flasks

Shake flasks were prepared containing sterile medium 2 (Table 2.2). Two or three colonies from freshly prepared JM 107/pQR 711 agar plates were added to the shake flasks aseptically. These were incubated for 12-16 hours on an orbital shaker (150 rpm) at 37 °C. These starter cultures were then used for inoculating larger shake flasks (500 mL) containing sterile batch medium 2. After 12-16 hours growth these shake flasks were used to inoculate the 5L fermentations.

2.2.2.2 Batch fermentations (5L)

The batch phase involved firstly a 5 L working volume batch fermentation in a 7L fermenter. The inoculum size was 500 mL into 4.5 L of medium 2 (Table 2.2), giving a total working volume of 5 L. The batch phase of the fermentation lasted for 20 hours by which time the entire carbon source (glycerol) was utilised. The 5 L batch-fermentations were used as inoculum for the 50 L fed-batch fermentations. The temperature of the 5L vessel was maintained at 37 °C. The pH of the culture was also maintained at 6.8 using 17 % ammonia solution and phosphoric acid (3M). The acid and the base were added automatically with a peristaltic pump. Over the 20-hour fermentation approximately 200 mL of ammonia solution (17 %) and 12 mL phosphoric acid (3M) were used.

2.2.2.3 Fed-batch fermentations (50 L)

In the 50 L fed-batch-fermentations the inoculum was a 5 L batch-fermentation into 35 L of the media. Giving an initial working volume of 40 L. The batch phase of the fermentation lasted approximately 2 hours by which time the dissolved oxygen dropped and glycerol feeding was started. For the fed-batch fermentation the feeding started at 11 mL h^{-1} with 200 g L^{-1} glycerol, 8.25 g L^{-1} $MgSO_4 \cdot 7H_2O$ and 0.125 g L^{-1} ampicillin (table 2.3). The magnesium sulphate and the glycerol were

sterilized together in the autoclave. The ampicillin was sterilized with a sterile 0.2 μm PSU Whatman filter.

The feeding was stopped just before 20 hours into the fermentations. After this time all of the 10 L feeding solution was added giving a total working volume of 50 L. The temperature of the 50 L vessel was maintained at 37 °C. The pH of the culture was maintained at 6.8 using 17 % ammonia solution and 3 M phosphoric acid. The acid and the base were added automatically with a peristaltic pump. Over the 22 hours fermentation approximately 1.75 L of Ammonia solution (17 %) and 0.2 L phosphoric acid (3 M) were used. In all fermentations sterile antifoam solution (PPG) was added manually when required. When the fermentation was completed and the nutrients were exhausted a sudden rise in the dissolved oxygen tension (DOT) was apparent. At this time a gradual fall in the oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER) was also observed. At the end of the fermentation the broth was harvested and moved to the downstream processing arena where the transketolase was obtained for experiments.

2.2.2.4 Implementation of the fed-batch fermentation

Transketolase was initially produced using batch fermentations. These batch fermentations were conducted using the defined media mentioned in Table 2.2 as well as media containing glycerol, tryptone, yeast extract and NaCl (LB media). However these batch fermentations suffered from low biomass. Based on the research previously published a fed-batch strategy was designed.

The fed-batch mode of operation that was calculated prolonged the fermentation at the same time kept the production of by-products to the minimum. A linear feed was calculated and implemented with the use of a pump to control the feed rate (Figure 2.2).

To implement a linear feed strategy a set of assumptions were made:

On starting the fermentation contains Glycerol at 5g L^{-1}.

The yield of biomass on glycerol (Y_{gly}) is 0.5 (0.5 grams of biomass from 1 gram glycerol).

Therefore at the start of feed (when initial carbon source is depleted) the broth contains 2.5 g/L of cells. Initial biomass X_0 = 2.5g/L.

The fed batch phase of the fermentation was estimated to take approximately 14 hours (t = 14).
The final biomass (X_t) desired was 22 g/L.

The specific growth rate (μ) threshold for *E. coli* is 0.35 h^{-1}. Above this level and there will be problems with lactate production in the fermentation (Han *et al.*, 1992).
Based on the assumptions the feed rate of glycerol to was calculated where:

$$X_0 = 2.5g/L$$
$$t = 14h$$
$$X_t = 22g/L$$
$$\mu = \ln\left(\frac{X_t}{X_0}\right)\Big/t = 0.155h^{-1}$$

The specific growth rate of 0.155 h^{-1} was lower than the threshold for *E. coli* (0.35 h^{-1}) (Han *et al.*, 1992). This meant that the feed rate could be manually adjusted slightly during the fermentation allowing control without causing major effect.

$\Delta X / \Delta t$ = 1.39 g/L/h of biomass
Y_{gly}=0.5
$\therefore$ ΔS = 2.79 g/L/h glycerol added in 14 hours

Final culture volume (V) = 50 L
Concentration of glycerol in feed solution (S_f) = 200 g/L

Glycerol feed rate (F) = 139 g / hr for 50 L culture.

Glycerol feed flowrate (V/S_f) = 0.698 L of feed solution added per hour.
Pump flowrate was calibrated and set at 11.6 mL/min.

Based on the calculations above the batch phase of the fermentation was allowed to take place until the limited carbon source (5 g/L) was depleted. This was indicated by a sudden rise in the DOT and a gradual decrease in OUR and CER. At this point the feed pump was switched on and the growth rate was monitored so that it did not

exceed the chosen limit. The feed rate was adequate to keep the OUR sustained at 50 mmol/Lhr. See Appendix II for details of typical fermentation results.

2.2.3 Enzyme recovery

After the completion of the fermentation the cells were harvested and the process of obtaining transketolase from the cells was initiated. Figure 2.3 outlines the downstream processing methods that were used to obtain the transketolase for subsequent biotransformations. The fermentation broth was firstly centrifuged to remove the cells from the media. At large fermentation volumes a Sorvall Super T 21 centrifuge (rotor SL-250T^2) was used for the collection of the cells from broth. This was achieved at 14500 rpm for 30 min at 4 °C. A large fraction of the cell paste was stored at –80 °C. The rest of the cell paste was resuspended in Tris-HCl buffer (50 mM, pH 7.6) to obtain a 1g/mL solution (based on wet cell paste weight). In large-scale purifications the resuspended cells were passed through a homogenisation step. A lab-60 APV Manton Gaulin homogeniser (Invensys APV, Crawley, West Sussex, UK) was used to disrupt the cells. A crude extract (10 L) was prepared by this high-pressure homogenisation (3 discrete passes at 400 bar, 60 l h^{-1}). After cell lysis a centrifugation step was undertaken. A Pennwalt 1P tubular bowl centrifuge was used for the collection of the cell debris from the homogenate. Achieved at 45000 rpm with a flow rate of 10 l h^{-1} this stage provided a semi-clarified mixture containing transketolase as well as other cell constitutes. The enzyme mixture obtained at this stage was not of the highest possible purity and in this investigation was labelled as crude transketolase (Tki).

For smaller scale purifications after the centrifugation and resuspension stages samples of the cell suspension were placed in 2.0 ml Eppendorf tubes. The cells were disrupted by sonication at an amplitude of 8 μm using a Soniprep 150 MSE (Sanyo, Crawley, West Sussex, UK) with a cycling programme of five times 10 second on/off bursts. During the sonication procedure the samples were placed in an ice bath to prevent them from overheating and losing of transketolase activity. The sonicated sample were finally centrifuged at 13000 rpm for 2 min (Biofuge 13, Heraeus Sepatech, Brentwood, Essex, UK) to remove cell debris and the resulting crude transketolase solution (Tki) was kept on ice before use in biotransformations.

In the large-scale Tk purifications other downstream routes were also tried. For cell breakage a Lab40 homogeniser (Invensys APV, Crawley, West Sussex, UK) was used after resuspension. Extracts (40 ml) were prepared by high-pressure homogenisation (2 passes at 1200 bar). These often resulted in very viscous transketolase mixtures that were not suitable for use in biotransformations. To solve this problem an alternative downstream route was investigated where the harvested broth was homogenised first using an APV Manton Gaulin Lab 60 homogeniser (3 discrete passes at 400 bar, 60 L/hr) followed by centrifugation. The impact of cell disruption in the media before centrifugation steps was not fully characterised in this study but resulted in less viscous mixtures.

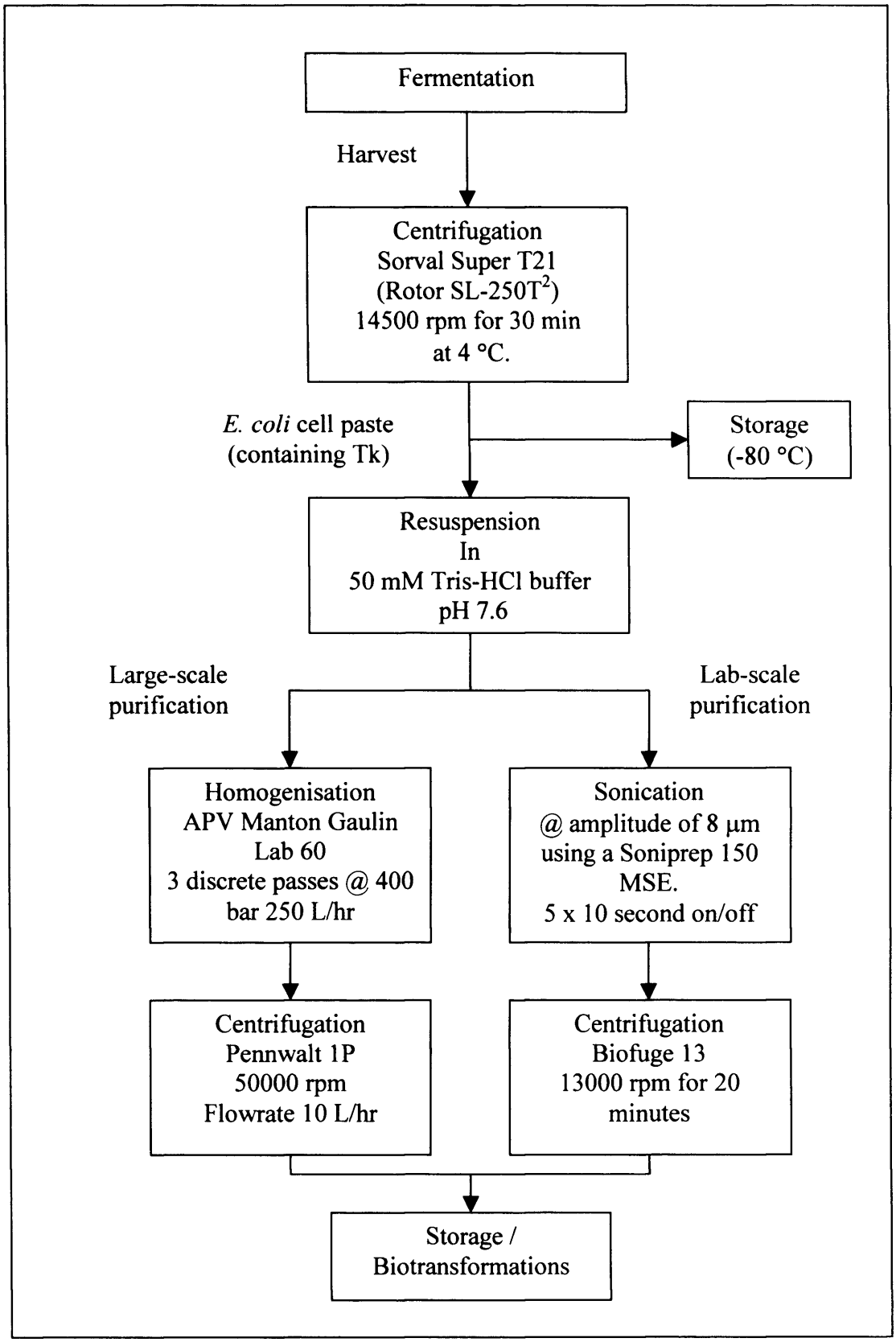


Figure 2.3 The recovery and purification of transketolase to obtain crude extract (Tki) showing the large scale and laboratory scale routes.

2.2.4 Storage and maintenance of transketolase

E. coli cell paste from fermentations containing transketolase were stored at –80 °C where the activity was maintained up to 3 months. Once the cells had been disrupted transketolase activity was rapidly lost. This loss of activity could be due to the oxidation of cystiene residues and the cleavage of the disulfide bridges in the protein. Protease mediated degradation could also be causing this loss of activity such as the presence of trypsin or chymotrypsin (serine proteases) (Gorbach, 1980). Therefore to avoid loss of activity the crude transketolase obtained was used in biotransformations immediately.

2.2.5 Chemical synthesis of lithium hydroxypyruvate monohydrate

8.39g of Lithium hydroxide ($LiOH.H_2O$, FW=41.96) was dissolved in 200mL water (1 M). Approximately 100 mL of this was added to a solution of bromopyruvic acid (1.67g, 10mmol) in 100mL water. This addition was carried out by slow auto-titration (Radiometer ABU91) so that the pH did not exceed 9.5. The reaction was stopped when the addition slowed down and came to a halt. At this stage a solid precipitate may form which must be re-dissolved by heating in a water bath (50 °C). The solution was otherwise clear and colourless. The formation of bromine out of the solution might result in a yellow mixture at this stage of the synthesis. Any yellow colouring may be removed by addition of activated charcoal (0.34 g). However no coloration was perceived at this stage of the process. The pH was adjusted to pH 5 by the addition of acetic acid (approximately 10 drops). The solution was concentrated in vacuo using a Büchi rotavapor R110 (Büchi Labortechnik AG, Flawil, Switzerland). The temperature of the water bath was set at 60-70 ° C. This was to remove as much water as possible. The solution was concentrated down to 20 %. The concentrated solution was cooled down to 0 ° C for 16 hours. This resulted in the formation of white crystals. At this stage the yellow supernatant was decanted and removed as much as possible without re-dissolving the crystals.

The resulting wet crystals (concentrated white suspension) weighed 3.82 grams and were assayed by HPLC to determine the exact amounts to add to each bioconversion.

2.3 Analytical methods

2.3.1 Spectrophotometric analysis

OD measurements at 670 nm (OD_{670}) were carried out using a Kontron Uvikon 922 variable wavelength spectrophotometer (Kontron Instruments Ltd, Watford, Hertfordshire, UK) in order to quantify biomass concentrations. Samples from fermentations were diluted and measured for optical density. To maintain accuracy the samples were diluted to the absorbance range 0.1-1.0. Fermentation broths were diluted with the original media used to account for the media absorbance. A calibration curve linking the OD_{670} measurement to the DCW measurement was constructed. The correlation between optical density and dry cell weight showed that dry cell weight DCW = 0.4 (OD). See Appendix III for a typical OD measurement.

2.3.2 Wet and dry cell weight (DCW)

To pre-dried and pre-weighed 2.2 ml Eppendorf tubes, 2 ml aliquots of cell culture were added and the tubes centrifuged at 13000 rpm for 2 minutes. The supernatant was discarded and the process was repeated for a further 2 ml aliquot and then a 1 ml aliquot in the same vial. After the final supernatant was discarded, the tubes were dried in an oven at 80 °C until they achieved a constant weight. The DCW of the culture was calculated by deducting the original Eppendorf weight from the final weight after the drying process noting that there were 2 mL aliquots. See Appendix IV for a typical DCW measurement.

2.3.3 Glycerol analysis

The glycerol in the fermentations was monitored at line. Samples from the fermentation were taken and assayed using the HPLC using a calibration curve obtained by running known concentrations of glycerol through the system. The conditions and method for the glycerol assay were identical to that of all HPLC analysis as described in Section 2.1.4.1. See Appendix V for a typical glycerol analysis using HPLC.

2.3.4 Measurement of protein

For the measurement of protein the Bio-Rad protein assay kit was used (Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK). The microassay procedure (accurate ≤ 25 μg/ ml) was implemented. Several dilutions of protein standard containing from 1 to 25 μg / ml were made from bovine serum albumin. Test samples were prepared from *E. coli* containing transketolase by resuspension in buffer (Tris-HCl 10 mM, pH 7.6) followed by sonication. The samples were diluted for the assay in buffer. 0.8 mL of standards and appropriately diluted, sonicated *E. coli* samples were placed in clean, dry cuvettes. 0.8 ml of buffer was placed in the "blank" test tube. 0.2 ml of Dye Reagent Concentrate (Bio-Rad) was added to all cuvettes. The cuvettes containing the samples and standards were mixed several times by gentle inversion (this avoided excess foaming). After a period of 5 minutes (at room temperature) the absorbance was measured at 595 nm versus reagent blank. The OD 595 was plotted versus concentration of standards and unknowns read from the standard curve. A standard curve was prepared each time the assay was preformed to improve accuracy. See Appendix VI for a typical Biorad protein analysis.

2.3.5 Calculation of transketolase activity

The activity of transketolase was measured based on the production of erythrulose from glycolaldehyde and hydroxypyruvate. This was due to the fact that transketolase has a high reaction rate on glycolaldehyde. Transketolase samples were pre-incubated with co-factors (TPP and Mg^{++}) prior to the addition of substrates in order to reduce the lag time before the reaction reached its maximum rate (Mitra *et al.*, 1999). To 1mL of transketolase samples glycolaldehyde and hydroxypyruvate were added to make up 100 mM concentrations. The biotransformations were monitored by taking samples every 5 minutes and assaying by HPLC. The initial rate of the reaction (within the first 20 minutes) was used to represent the activity of transketolase. This activity was calculated in terms of μmoles per minute per mL of transketolase. 1 mL of Transketolase typically produced 2.35 mg mL Erythrulose in 20 minutes (1 U mL^{-1}). The specific activity was calculated using the Biorad protein assay. This resulted in the specific activity of 0.12 U mg^{-1}. See Appendix VII for a typical calculation of Tk activity.

3 The multi-enzymatic characterisation of xylulose 5-phosphate preparation.

3.1 Introduction

The following chapter was aimed at characterising the multi-enzymatic preparation of xylulose 5-phosphate (X5P). It was aimed to gain as much information as possible on the characteristics of this complex system. Strategically it is preferable that this is carried out with the minimum number of steps. Thus a logical approach is taken to the order in which these experiments are carried out (Section 1.8.1).

The multi-enzymatic production of X5P reaction scheme can be split into three sections (Figure 3.1). Firstly the fructose 1,6 bisphosphate (Fru 1,6 BP) system is analysed in depth to identify the major constraints of the system. Secondly reaction components are tested with respect of stability. This identifies the particular properties of each component and how they impact the system as a whole. The importance of substrate levels in the reaction is addressed in the preparation of X5P from glyceraldehyde 3-phosphate (G3P) as well as from dihydroxyacetone phosphate (DHAP). Accordingly data are collected on the toxic effects of the components on *E. coli* transketolase. Finally the impact of compromising levels of enzymes in the reaction is described.

The results of all the experiments are reported in the form of operating maps / windows for the reaction system. Using these techniques the multi-enzymatic system is fully characterised in the smallest time frame. These experiments together with the data gathered help to provide a logical step-by-step plan for the characterisation of a multi-enzymatic system and lay the groundwork for subsequent further analysis of the system. The results from this chapter will aid the multi-enzymatic process development defining constraints and guidelines, which can be used to rule out or eliminate unattractive process options at this early stage.

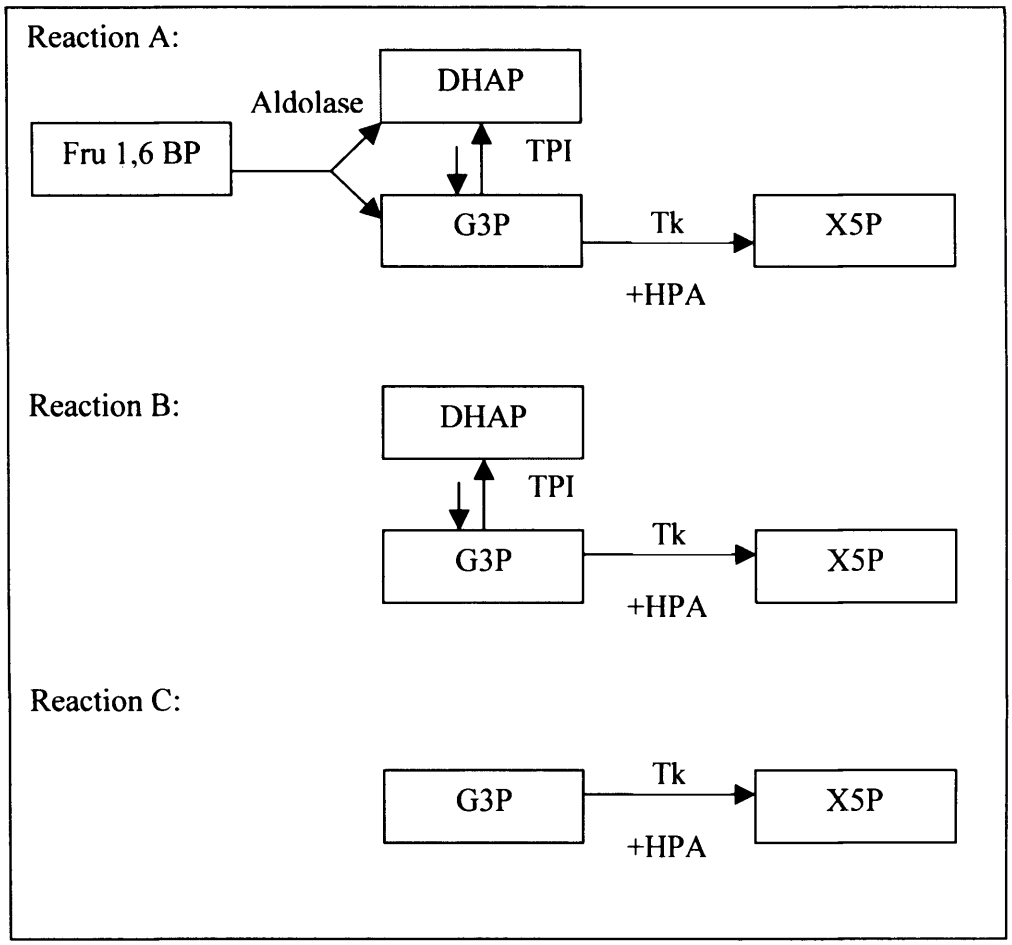


Figure 3.1 Examining alternative routes to the multi-enzymatic preparation of X5P. By breaking down the complex reaction A it is possible to examine the individual steps more accurately. Reaction B starts with DHAP and reaction C starts with G3P as a substrate.

3.2 Materials and methods

3.2.1 Biotransformations

Biotransformations were conducted at small-scale using glass baffled bioreactors (5mL-100mL) with agitation control where the pH was controlled using auto titration pH stat apparatus (Radiometer Ltd. West Crawley, Sussex, UK). The pH was controlled using HCl (0.1-1M HCl) and temperature kept at 25 °C. The reactions were initiated by the addition of starting substrate to the reactor containing biocatalysts, HPA and co-factors TPP (2.4 mM) and Mg^{++} (0.9 mM). To reduce oxidation of the biocatalyst mercaptoethanol (10 mM) was added to the reaction mixture. Transketolase (from fermentations) was added to requirement from Tki source 1 U mL^{-1} (0.12 U mg^{-1}) or Tkp source 0.5 U mg^{-1} protein. TPI was added as required from a source with activity of 5000 U mg^{-1} protein (Sigma-Aldrich, Poole, Dorset, UK). The biotransformation all used transketolase from fermentation as a source and progress was recorded by taking samples regularly and analysing using the HPLC method described in Section 2.1.4.

3.2.2 Production of X5P using Fru1,6BP

The preparation of X5P form Fru1,6BP was adapted from the methods described in the literature (Zimmermann *et al.*, 1999). An aqueous solution (250mL) containing Fru1,6BP (from rabbit muscle) (10 mM), HPA (20 mM), $MgCl_2(H_2O)_6$ (0.9 mM), TPP (2.4 mM) was adjusted to pH 7.0 using the pH stat (Radiometer Ltd., West Crawley, Sussex, UK) with 0.5 M HCl. The reaction was started by the addition of FruA (72 U), TPI (125 U) and Tk (200 U). Further HPA was added at 8 hrs (330 mg) and at 24 hrs (330 mg). The reaction was monitored using the pH stat and by taking samples and testing using the HPLC method described in Section 2.1.4 to calculate the level of X5P and HPA in the solution.

3.2.3 Effects of pH on substrates and product

5 mL solutions of hydroxypyruvic acid (HPA), glyceraldehyde 3-phosphate (G3P), dihydroxyacetone phosphate (DHAP) and xylulose 5- phosphate (X5P) were prepared up to 500 mM. The pH was adjusted to range 6.5 – 9.0 using 0.5M HCl or 0.5 mM NaOH and maintained at that value over 6 hours using a pH stat.

Temperature was controlled at 25 °C. Samples were removed and analysed by HPLC to calculate the level of compound remaining as a percentage or fraction of the original concentration.

3.2.4 Levels of substrate

Controlled reactions were carried out using a range of substrate concentrations using the methods described in Section 3.2.1. Varying concentrations of starting substrates (G3P, DHAP and HPA) were applied to controlled levels of biocatalyst. Samples were taken at regular intervals and assayed for X5P and HPA using the HPLC.

3.2.5 Effects of substrates on transketolase

These measurements were made under the most favourable conditions for the enzyme. The enzyme was pre-incubated with 0.9 mM Mg^{++}, 2.4 mM TPP and reducing agent mercaptoethanol (10mM). pH was at 7.0 and the temperature at 25 °C. Various concentrations of components were incubated together with transketolase individually for 6 hours at which point the samples were tested for residual activity, calculated and presented as a percentage of the starting activity. The loss of activity was calculated by presenting the final activity as a percentage of the initial activity considering the loss of activity in the control (Tk and the co-factors without the substrates or product).

3.2.6 Levels of enzyme

Controlled reactions were carried out using varying concentrations of Tk and TPI. The reactions were conducted using the methods described in Section 3.2.1. The effects of changing levels of TPI in the reaction was tested by adding TPI to reactions at 200 U ml^{-1} , 40 U mL^{-1} and 20 U mL^{-1} to reactions containing 15 mM DHAP, 30 mM DHAP and 50 mM DHAP. Transketolase levels were kept constant throughout (1 U mL^{-1}). The reactions were tested for X5P formation using HPLC. To test the effects of increasing Tk levels on the reaction various levels of Tkp (1, 4, 8 and 12 U mL^{-1}) were added to reactions containing 30mM G3P. The X5P production was monitored by taking regular samples for HPLC analysis.

3.3 Results

3.3.1 The production of X5P using fru1,6 BP

The first step towards characterising a new system is to become familiarised with its major constraints. This involves the determination of the characteristics of the various components of the bioprocess – both individually and their interaction with each other (Mitra *et al.*, 1998). Previous studies on the biocatalytic production of xylulose 5-phosphate have described the production using fructose 1,6 bisphosphate (Sprenger *et al.*, 1994). To confirm the previous findings this biotransformation was conducted using the techniques described in literature. This time however the use of HPLC and electrochemical detection allowed a more detailed analysis of the system. This experiment was also the first step towards becoming familiar with this model reaction. Sprenger and co-workers have previously described how fructose aldolase is added to fructose 1,6 bisphosphate to break it down to DHAP and G3P (Sprenger *et al.*, 1999). The G3P in the presence of HPA and transketolase was then converted to X5P. The reaction mixture included transketolase cofactors (TPP and Mg^{++}). Although the data provided indicated high conversion (97%) the reaction suffered a lag phase of 3 hours and took 48 hours to reach completion.

In this preliminary experiment the same methodology was applied to produce X5P. The aim of the data gathered was to provide as much information on the constraints of the reaction and show previously unknown data such as HPA usage. Figure 3.2 shows the multi-enzymatic production of X5P and the levels of HPA in the reaction mixture. As shown in this reaction profile it was necessary to add further HPA. In previously described methods HPA was also re-added to the reaction mixture at intervals. Further transketolase was also added to the reaction. Although the reasons for this were not clearly explained in the literature this was presumably due to the instability of transketolase and HPA. When replicating the procedure here it was clear that HPA is lost at a higher rate than X5P production (Figure 3.2). HPA in this case was added to the system at 8hrs and after 24 hours in accordance with the Sprenger protocol. Table 3.1 shows the results obtained here in comparison with those gathered from the Sprenger published material. The data in this

experiment is closely comparable to those published by Sprenger *et al.*, 1999. However there are some important differences that must be noted. The total X5P produced using this method was shown by HPLC electrochemical detection to be 21% less than that indicated by published data. It is also clear that there is unused and residual substrate in the form of HPA (4.62 mM). This was the first time that the level of HPA was described fully using HPLC detection in this reaction. The results here showed clearly that the substrate is not completely converted to product. The results also indicated a considerable lag phase of 7 hours where no X5P was formed. This lag phase was probably attributed to the reaction being limited by the aldolase cleaving fructose 1,6 bisphosphate into DHAP and G3P.

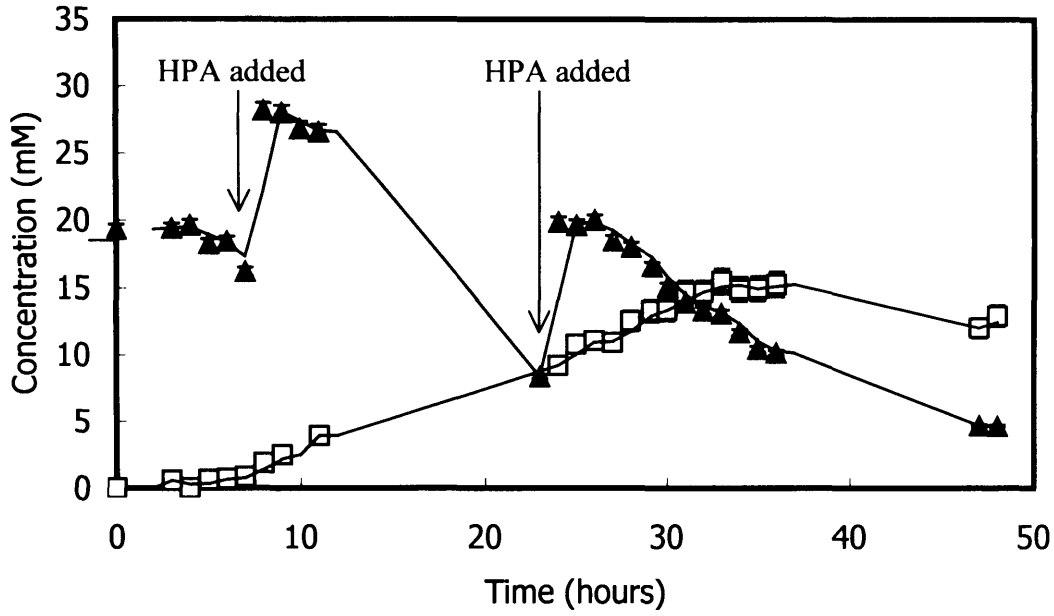


Figure 3.2 The reaction profile for the preparation of X5P (⊣□⊢) from Fru 1,6 BP and HPA (⊣▲⊢). The reaction was initiated with the addition of TK and arrows on the plot indicate when further HPA was added.

The results in this experiment indicated clearly that this multi-enzymatic system suffers from inefficiency. The 48 hour period in which the production takes place accordingly lowers the systems productivity. Consequently also substrate and biocatalyst need to be added during the process. As with previously published results the initial concentration of HPA (20 mM) seems lower than that needed to

drive the transketolase reaction to completion. This may be the reason for the poor thermodynamics. The results showed that HPA suffers from degradation (54.9 %). This can be again attributed to the long reaction time.

	Total X5P produced (mM)	X5P (g/L)	Total HCl used (mL)	HPA converted to X5P (mM)	HPA remaining In Product stream (mM)	Productivity (g/L/hr)	Maximum reaction rate (g/L/hr)
Sprenger *et al.*, 1999	19.41	4.47	8.0	19.41	unknown	0.093	0.16
Reaction with JM107/pQr 711 Tk	15.24	3.51	10.2	15.24	4.62	0.074	0.10

Table 3.1 Showing a comparison between published data by Sprenger *et al.*, 1999 and reaction run using transketolase from *E. coli* JM107 pQR 711.

So far the major constraints of this system based on the results are:

1. Substrate degradation (the stability of substrate must be fully characterised).
2. Low reaction productivity. This was due to poor kinetics and thermodynamics. There was also a lag phase as the system is probably limited by aldolase. The reaction time was shown to be too long.
3. Further biocatalyst was needed (perhaps due to the effects of reaction components on transketolase).

Based on the results it was possible to move to the next stage of decomposing the system meaning that the reaction was no longer started with Fru 1,6 BP. The subsequent experiments were focused on characterising the system starting from G3P and DHAP (reaction b and c in Figure 3.1). In the following experiments the characteristics of these biotransformations were investigated. This was to determine the process options and in particular to build an integrated systematic characterisation approach for any given multi-enzymatic synthesis.

3.3.2 Substrate stability

As indicated by the results in Section 3.3 one of the major constraints on the X5P system is possible substrate degradation. It was important to analyse the major components of the system to clearly show how susceptible they are to degradation.

3.3.2.1 Effects of pH on substrates and product

The incubation of substrates and product over a range of pH values in the presence of cofactors indicated that all components were stable in acid but showed a marked instability under alkaline conditions at 25 °C. Figure 3.3 indicates clearly how stable the reaction components are with increasing pH. All components/educts were stable to different degrees. The stability of all components was shown to be affected by pH. There was a need for further in-depth examination to identify the exact nature of this instability. HPA degraded to a higher degree as pH increased. This data confirms the initial results published made by Mitra and co-workers on HPA pH stability (Mirta *et al.*, 1998). The previously unknown information on G3P, DHAP and X5P is also shown in Figure 3.3. The stability of each component is indicated using the same methods as those for HPA (Section 3.2.4). The assumption was made that high molarity is the worst-case scenario as stability has been shown to be concentration dependent (Mitra *et al.*, 1998) and for this reason the components were tested at high concentrations. G3P was the most sensitive component to pH. It degraded more readily even at pH 6.5. DHAP followed a similar trend and profile to G3P but degrades to a lesser extent than G3P.

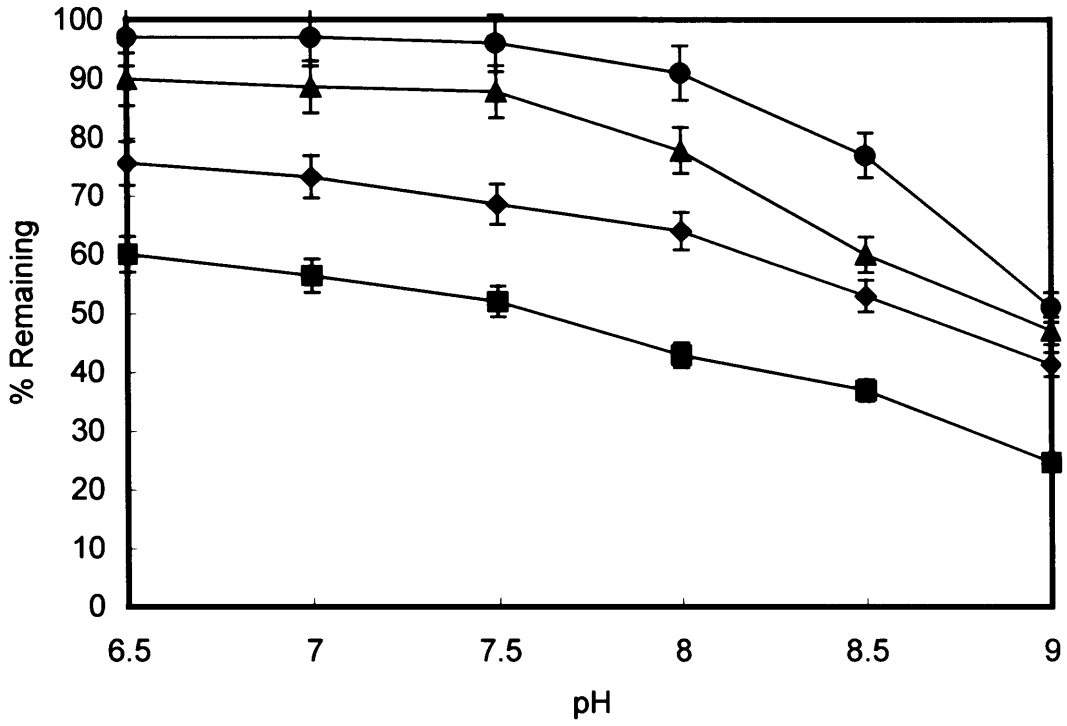


Figure 3.3 The stability of reaction components HPA (—●—), X5P (—▲—), DHAP (—◆—) and G3P (—■—) at lab scale concentrations (0.5 M) in terms of pH (6.5-9.0) indicated by the percentage remaining after a 6-hour incubation period. Error bars calculated based on triplicate analysis.

The product X5P was more stable than G3P and DHAP but greatly effected as pH shifted above 7.0. At pH 7.0 the results showed that G3P suffers a loss of 43.6 % while DHAP suffers a loss of 26.7%. The results gathered here did not provide a full picture of how the substrates degrade during the 6 hour time period. This was considered important as it further characterised the components. Figure 3.4 shows in greater depth how G3P degrades within the 6-hour time frame. It was shown by the results that G3P suffers the greatest loss at high concentration and high pH (62 % loss). Even at low concentration and low pH there is a 36 % loss. The results showed that there is considerable degradation very early on in the subsequent

possible biotransformations. There was a minimum of 15 % loss in the first 2 hours of incubation.

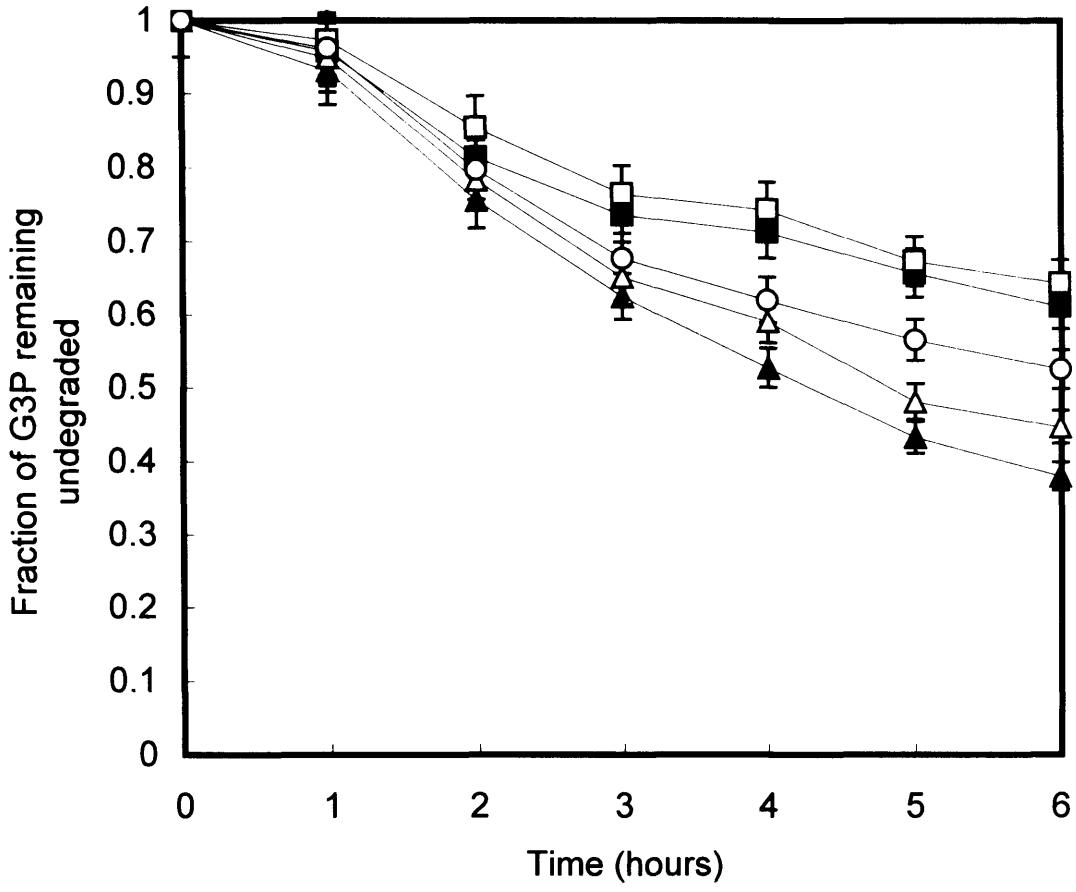


Figure 3.4 The stability of G3P indicated as a fraction of the starting concentration at 30mM, pH 6.5 (-□-), 150mM, pH 6.5 (-■-), 30mM, pH 7.8 (-○-), 30mM, pH 8.5 (-△-) and 150mM, pH 8.5 (-▲-). Error bars calculated based on triplicate analysis.

A similar analysis was carried out on DHAP. The results are shown in Figure 3.5. Here the substrate was less susceptible to degradation. The results indicate that in the first 3 hours of incubation there was relatively little substrate lost even at a high concentration and pH (12 % lost in 3 hours at 150 mM and pH 9.0). Figure 3.5 shows clearly the complete range of DHAP stability.

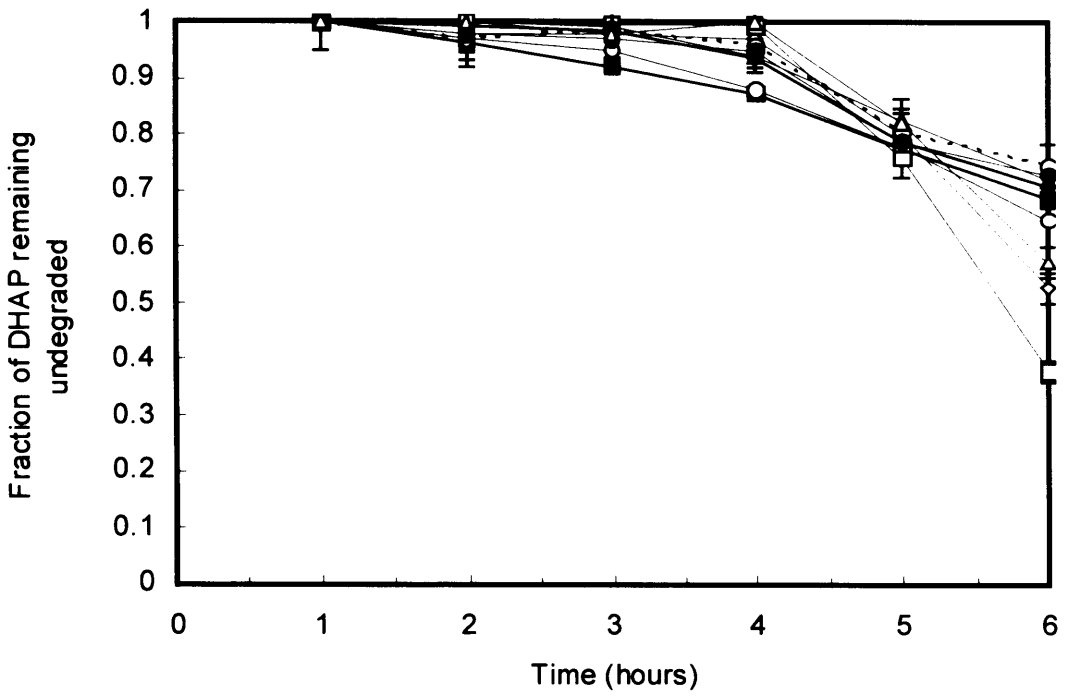


Figure 3.5 The stability of DHAP indicated as a fraction of the starting concentration at 30mM, pH 6.5 (---○---), pH 7.0 (—●—), pH 8.5 (—▲—), pH 9.0 (—◆—), at 100mM, pH 7.8 (—△—) and at 150mM, pH 6.5 (—■—), pH 7.0 (—○—) pH 8.5 (—◇—) and pH 9.0 (—□—). Error bars are calculated based on a triplicate analysis.

By incubating a range of DHAP concentrations in different pH environments it was shown that within a 6 hour time frame degradation took place. The results were pooled to show the stability range of DHAP (Figure 3.5). Unlike the G3P stability results, DHAP was more stable at high concentrations and in alkaline environments. The results indicated when the dissociation of the phosphate occurred at different pH values and concentrations. At high pH and high concentrations degradation did not occur for up to 3 hours but then was shown to be rapid thereafter. At low concentrations degradation started earlier (2 hours) but continued at a slower rate. Although this information was indicative of the component characteristics it was not considered necessary to explore further at this stage of characterisation. Only

data relevant to process design and selection should be gathered (Blayer *et al.*, 1996). The results at this stage were good enough to show a comparison between the two main substrates (Figures 3.6 and 3.7).

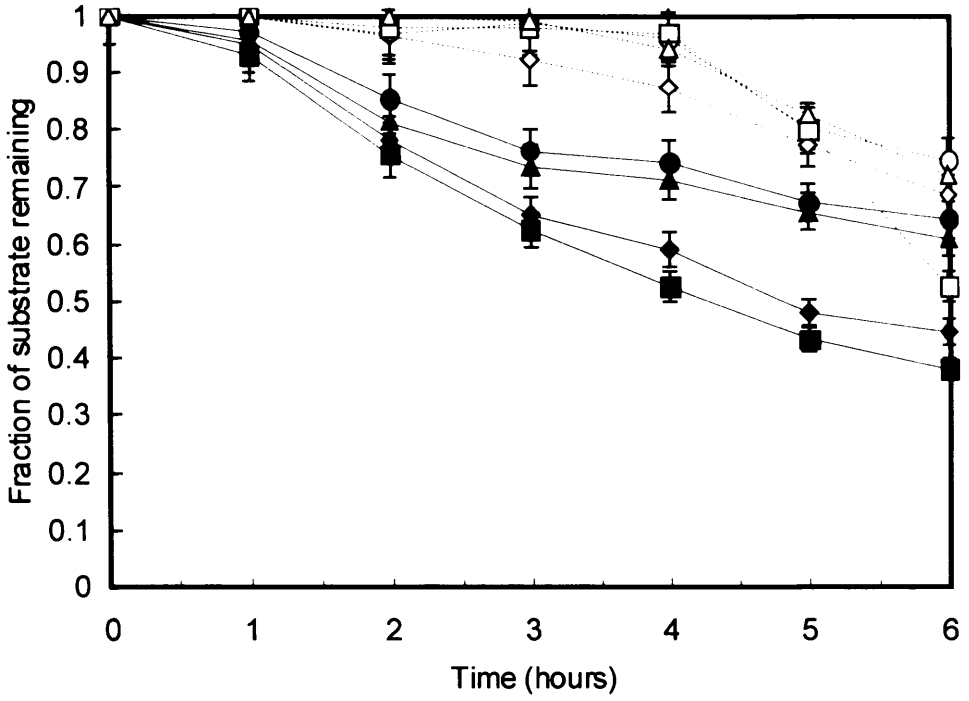


Figure 3.6 A comparison of G3P and DHAP stability indicated by the fraction of DHAP remaining at 30mM pH 6.5 (---○---), 30mM pH 8.5 (---△---), 150mM pH 6.5 (---◇---), 150mM pH 8.5 (---□---) and the fraction of G3P remaining at 30 mM pH 6.5 (—●—), 30mM pH 8.5 (—◆—), 150mM pH 6.5 (—▲—) and 150 mM pH 8.5 (—■—).

Making a comparison of substrates stabilities was important in showing the eventual process. The data provided clues with regards to the process limits and starting substrate. From Figure 3.6 it was clear that G3P at high pH and concentration suffered losses of 62% in 6 hours whilst DHAP suffered losses of 48 %. Under milder conditions and lower concentration the same effect was observed with G3P losing 35 % and DHAP losing 25 % after 6 hours.

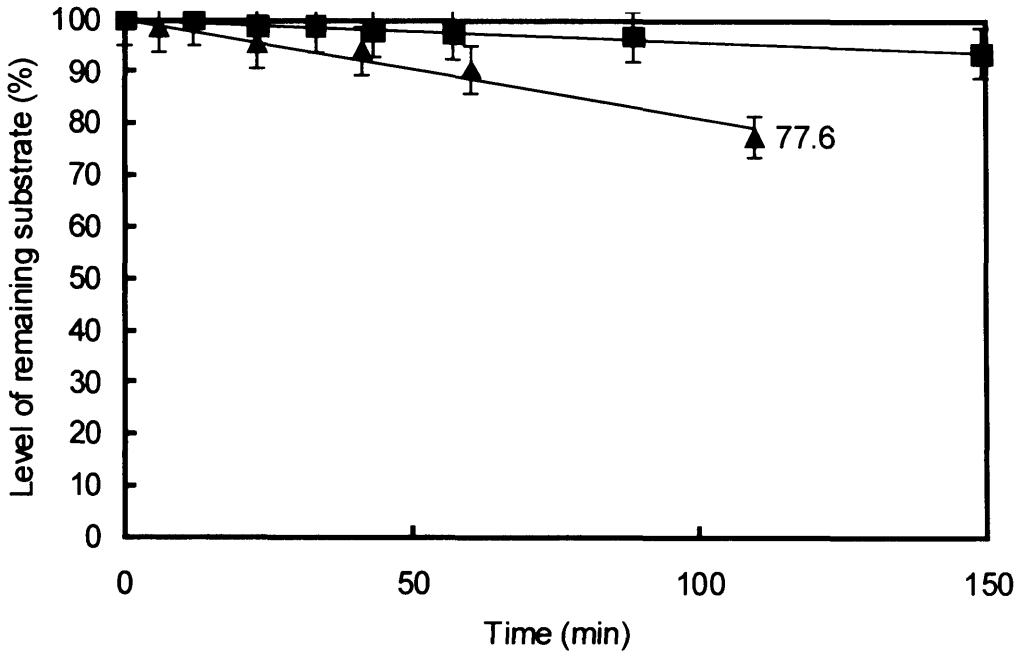


Figure 3.7 The stability of DHAP (—■—) and G3P (—▲—) in typical reaction conditions (25 °C and pH 7.0) at 30 mM concentration. Error bars calculated from duplicate analysis.

Figure 3.7 shows that the rate of DHAP degradation initially is 2.52 % per hour. At process scale 30 mM DHAP corresponds to 3.12 g L^{-1}. A 2.5 % loss per hour results in the loss of 0.079g DHAP in one hour or 0.47 g of substrate lost in 6 hours. G3P on the other hand suffered a loss of 11.4 % in the first hour. The stability of DHAP in comparison to G3P is higher. G3P is a relatively unstable intermediate and sensitive to pH changes. Running the reaction starting with G3P as a substrate will mean a more sensitive process, very much affected by pH shifts. DHAP in comparison is very stable this was shown to be the case when the two substrates are kept at typical reaction conditions (pH 7.0).

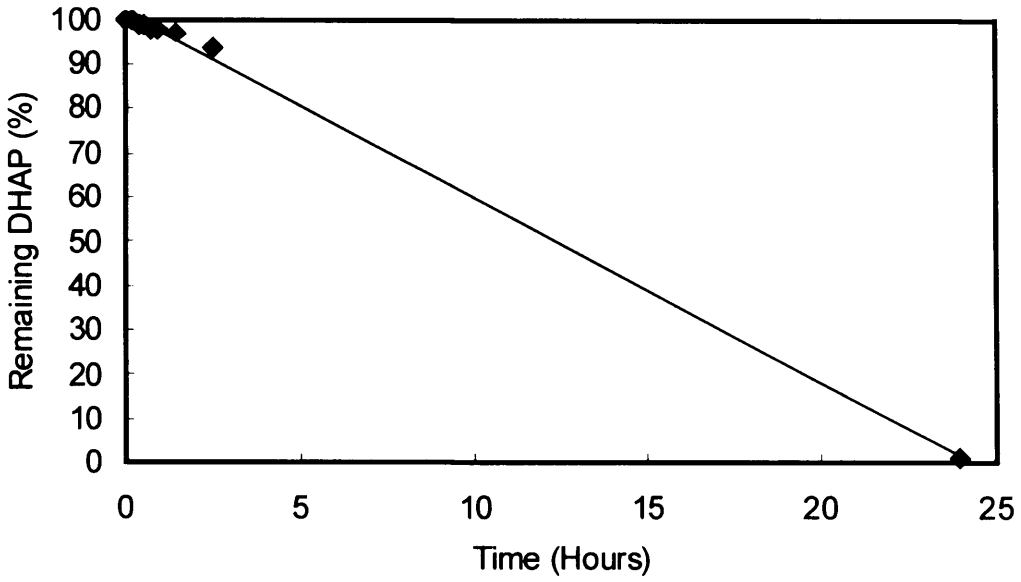


Figure 3.8 The degradation of 30mM DHAP (—◆—) over a 24-hour time scale.

However none of the components are stable over long periods of time where DHAP being one of the most stable compounds breaks down completely in 24 hours (Figure 3.8). This degradation was due to pH of 7.0 and temperature of 25 °C. These are typical working conditions of the model multi-enzymatic system. For reactions running for long periods of over 6 hours there is a need to supply further DHAP substrate. However DHAP lasts longer than G3P over long periods. As shown in previous figures. G3P also degraded fully within 24 hours (data not shown) at these conditions. The stability of holotransketolase is known to be maintained between pH 5.5-10.0 and the optimal pH range for activity is 7.0-7.5 (Mitra *et al.*, 1998). TPI activity was also maintained at this pH. Taking all this into consideration pH control comes to the forefront of priorities when running batch biotransformations involving these components. In this characterisation the first important consideration was the properties of the substrates and product. By monitoring the degradation of DHAP, G3P, HPA and X5P of a range of concentrations in different pH environments for periods of 6 hours it was possible

to gather some important data on the properties of each component. The results indicated a general decrease in the detectable concentration of the components with time. This effect was magnified with increasing pH. Substrates and product were shown to be sensitive to alkaline environments. This was an important observation with respect to G3P, a high value-low concentration component of the reaction and the most susceptible to pH change. The degradation of the product at pH 7.0 was 11.4% (Figure 3.3). This reiterates the importance of time scale and that reactions times must be kept to the minimum further explaining the drop in product concentration seen after 35 hours (Figure 3.2). Typically HPA also suffered losses due to instability at high pH. The results gathered on HPA reconfirmed those previously published in literature. Detail on the physical properties of G3P and DHAP was lacking in literature particularly with regards to their pH stability. Further in-depth analysis of the pH stability of G3P and DHAP enabled a direct comparison of the two substrates. It was discovered that G3P started to degrade immediately in solution. DHAP on the other hand was more stable in solution with no significant loss for up to 4 hours. The results gathered in experiments for Figures 3.4, 3.5 and 3.6 provided great insight into how the molecules behave in solution in different pH environments. For the purposes of scaleup it was important to distinguish whether the degradation was concentration dependent or not. By analysing data from Figures 3.3, 3.4 and 3.5 it was clear that the stability of DHAP and G3P is concentration dependent up to 150 mM and at higher pH values. After this point the degradation continues with time and it is not shown beyond 6hrs. This information is important as it conveys that if a model process is to be run at low concentration particular attention must be given to pH. At low concentration the substrates are more susceptible to pH changes. Based on the data gathered here G3P is unstable to a higher degree than DHAP even at low pH and low concentrations. This was probably due to the phosphate group on G3P being lost in solution.

More investigation is needed to study the concentration effect. Particularly for G3P at higher concentrations (500mM and above). These data points were impossible at this time due to low concentrations and high value of G3P available on the market and subsequently available for research here. The results gathered on stability imposed a constraint on the maximum pH of the model process. The lower pH limit of the reaction was defined by the enzyme stability and pH optimum. The crude *E.*

coli transketolase (Tki) derived from fermentations in this work has been shown to be irreversibly denatured at all pH values less than 6.5 and the pH optimum is in the range 7.0-7.5 (Mitra *et al.*, 1998). At this point enough data was gathered to place limits on the final process. This was deemed to be the distinct indicator for moving on to the next stage of experimentation.

3.3.3 Levels of Substrate

A series of biotransformations were performed at a range of substrate concentrations with a pH stat system to maintain a constant pH. It was important to gain process information with regards to each substrate. Here the biocatalyst levels were kept constant to study the kinetics and thermodynamics using increasing substrate concentrations. The potential process was simulated in an agitated vessel with 5 mL reaction volume. The primary aim of these experiments was to establish the ideal substrate conditions needed for yielding an increased quantity of product in the reaction as well as fast reaction rates. The reactions were all monitored for increasing product X5P and decreasing levels of substrate HPA. It was considered inaccurate to view the reactions in terms of DHAP and G3P. The HPLC method used in this research did detect these components but the peaks were often very close together and poor resolution was experienced. With HPA being the key substrate in X5P production. Experiments determining its requirements in biotransformations were amongst the first.

3.3.3.1 Identifying the required level of HPA in reactions

Sets of unbuffered biotransformations starting with a range of HPA concentrations were carried out. The reaction profiles monitoring the decline in substrate (HPA) and the increase in product (X5P) are given in Figure 3.9. For a highly productive and efficient biotransformation process the time is ideally kept to the minimum. It was apparent from the profiles that the time-scale for the reactions were cut down drastically by decomposing the multi-enzymatic reaction (approximately 8 times). In literature X5P had not previously been produced in this manner. Figure 3.9 illustrates that the lag phase experienced in reactions involving fructose 1,6 bisphosphate is eliminated and the total reaction time is within 6 hours. This was achieved by using DHAP as a starting substrate. In these biotransformation

particular attention was given to the ratio of one substrate to the next. For example in a system starting with DHAP and HPA it was imperative to calculate to what ratios should these two components be present in the reaction vessel. In previously run systems the level of HPA had been equal to that of G3P (Zimmermann *et al.*, 1999). The results here stressed that HPA must be in excess to drive the reaction towards completion. The reaction profiles in Figure 3.9 show that the product yield and rate increases directly as the ratio of HPA to DHAP increases. The best conversion is achieved when the ratio of HPA to DHAP is approximately 2:1. From the reaction profiles in Figure 3.9 it was gathered that the rate of reaction increases with HPA to DHAP ratio. It was noted however that this reaction rate could have been limited by the TPI reaction and further experimentation was needed to clarify this. By looking at the reaction scheme it was clear that even with excess HPA present there needed to be enough TPI in the system to convert DHAP to G3P. This G3P was necessary for the second reaction to reach completion.

At this stage the results highlighted that characterisation of multi-enzymatic interactions should not be carried out disregarding either the substrates or the enzymes. Experiments were carried out with full attention to all aspects of the system. Biocatalysts and substrates needed to be characterised alongside each other and not completely independently.

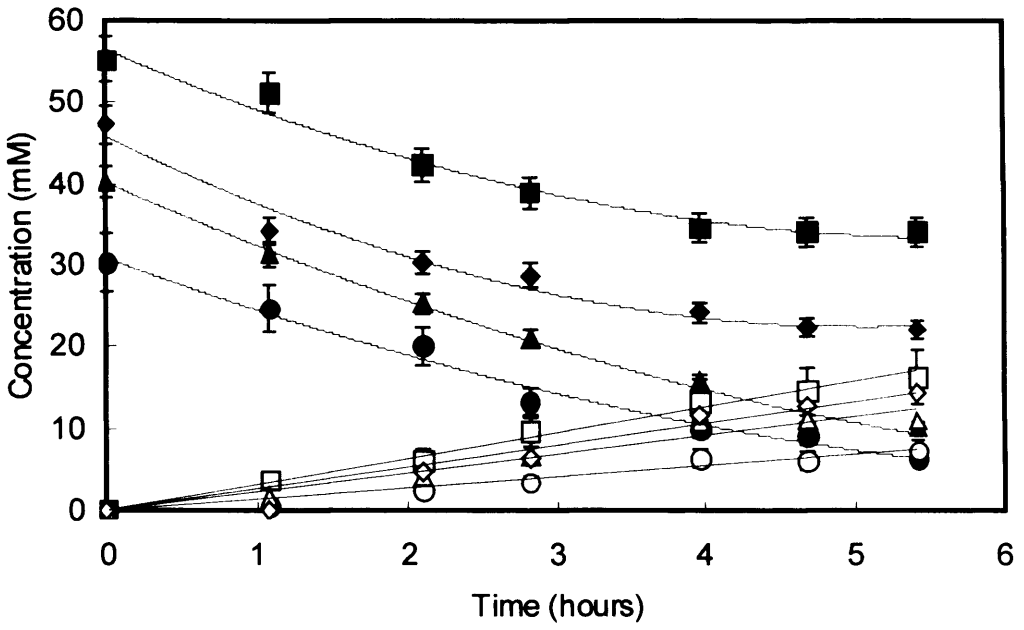


Figure 3.9 The impact of changing HPA levels on the preparation of X5P from 30mM DHAP indicated by the detected HPA levels, 60 mM (—■—), 50 mM (—◆—), 40 mM (—▲—), 30 mM (—●—) and corresponding X5P levels from 30 mM HPA (—○—), 40 mM HPA (—△—), 50 mM HPA (—◇—) and 60 mM HPA (—□—). Reaction conditions were kept at 25 °C and pH 7.0.

These experiments were necessary to determine the optimum levels of HPA at which the biotransformations should be operated. The transketolase source was a solution of the enzyme obtained from crude extract. The relatively low return on substrate might be attributed to this. It was possible to analyse the reaction profiles with regards to the rates of reaction. The highest rate achieved was 0.06 mM min^{-1} with a HPA to DHAP ratio of 2:1. The yield of product on substrate [X5P]/[DHAP] was calculated in terms of concentration (mM) to be 0.48 at best. The initial rates of reactions were calculated in all cases to be normalised based on the amount of transketolase (specific activity). These values were pooled together to provide a better overall picture of the substrate issue (Table 3.2).

3.3.3.2 Production of X5P from G3P

In this model multi-enzymatic synthesis all reactions lead to the transketolase catalysed conversion of HPA and G3P to X5P. It was therefore considered vitally important to understand more about the impact of G3P as a substrate on X5P production. Biotransformations were monitored with G3P as a starting substrate. The example shown in Figure 3.10 illustrates that the reactions starting with G3P took place in a very short time. Almost 60 % of the substrate was converted into product within the first hour.

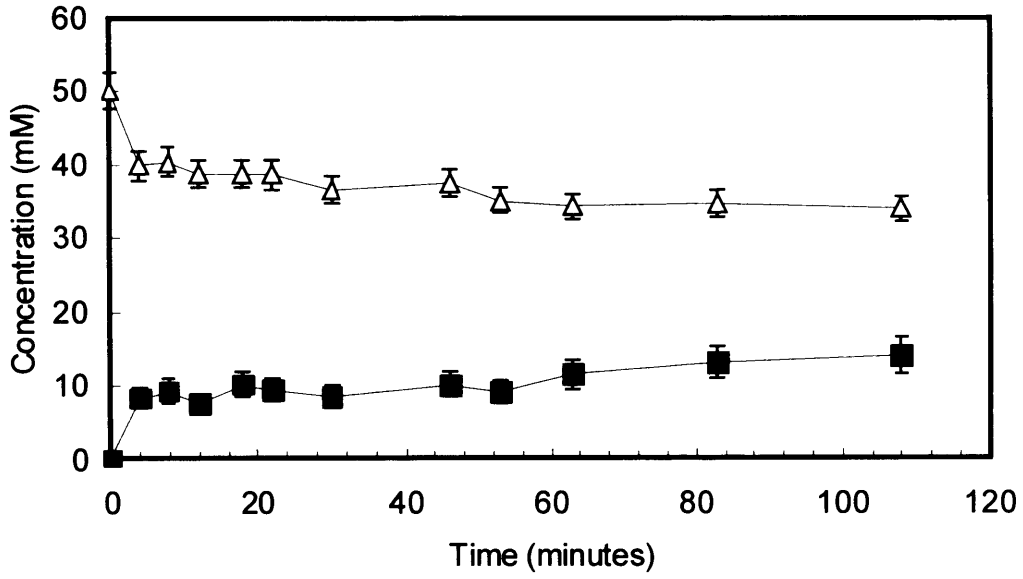


Figure 3.10 Preparation of X5P from 15 mM G3P using Tkp (1 U mL^{-1}). Showing the increase in product concentration X5P (–■–) and the depleting HPA (–△–) as ketol donor.

The overall yield of product from G3P in this case was above 80%. None of the reactions showed 100% conversion. This was probably due to the crude source of transketolase used or as a result of inhibition. Experiments incorporating pure transketolase resulted in better conversions (<90%) in the case of G3P. In the case of DHAP this yield was lower (approx 85%) even with pure biocatalyst used. The results indicated some equilibrium and yield issues involved in this reaction. These

yield and equilibrium problems were probably related to the ratios of TPI and Tk in the reaction vessel.

3.3.3.3 Production of X5P from DHAP

Biotransformations were conducted with varying levels of DHAP as a starting substrate. No G3P was added to these reactions whatsoever. This meant that DHAP was first converted to G3P by TPI. Transketolase was then responsible for converting G3P into the desired product X5P.

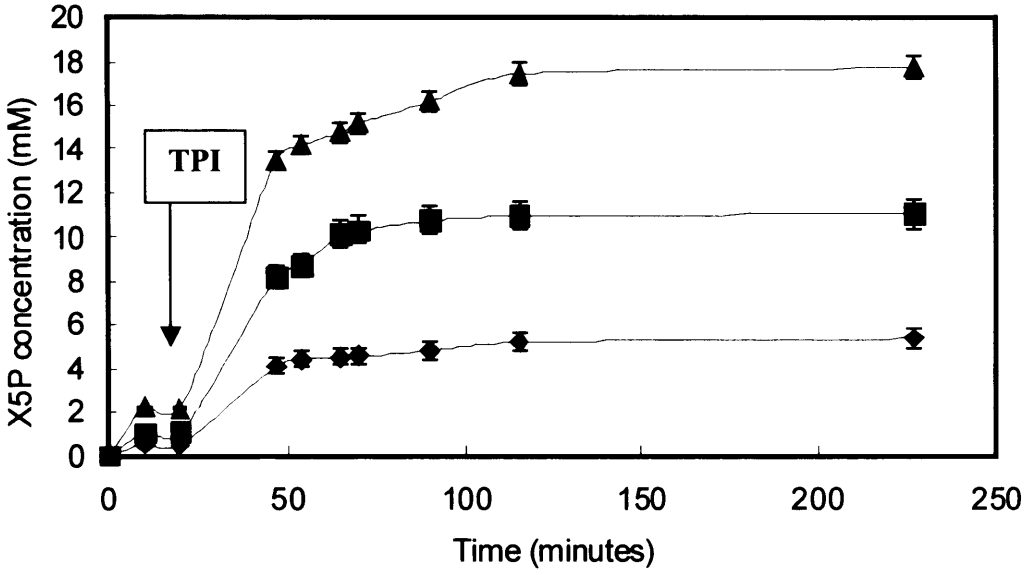


Figure 3.11 The preparation of X5P from DHAP at 5 mM (—◆—), 15 mM (—■—) and 20 mM (—▲—) initial concentrations with arrow showing TPI (20 U/mL) added at 20 minutes.

Reactions starting with DHAP as a starting substrate are dependent on the presence of TPI for completion. The results highlighted in Figure 3.11 show that there was a small amount of product formed due to the TPI present in the semi-purified transketolase. With the addition of TPI at 20 minutes the production was at a much higher rate and yield. The profiles of these reactions, depicting X5P concentrations determined by the HPLC assay showed that the initial rates of the reaction increased with increasing DHAP concentration but this increase was not observed to be linear.

The fact that the reaction rate did not increase linearly was a strong indication that the X5P reaction is limited by TPI.

3.3.3.4 Comparison of using DHAP and G3P

Biotransformations were carried out simultaneously to make a direct comparison of the different starting substrates. The results showed that DHAP reactions suffered from a lower yield and in some cases slower reaction rates than G3P reactions. This difference was not significant at small scale but must be considered for scale-up of the model process. Figures 3.12 and 3.13 are good examples of this phenomenon. At 50 mM concentrations (Figure 3.12) the initial rates for the two profiles are identical. The yield of [X5P]/[G3P] was shown to be 0.49 whereas the yield of [X5P]/[DHAP] was 0.41. There was a difference of 17% in yield at small scale.

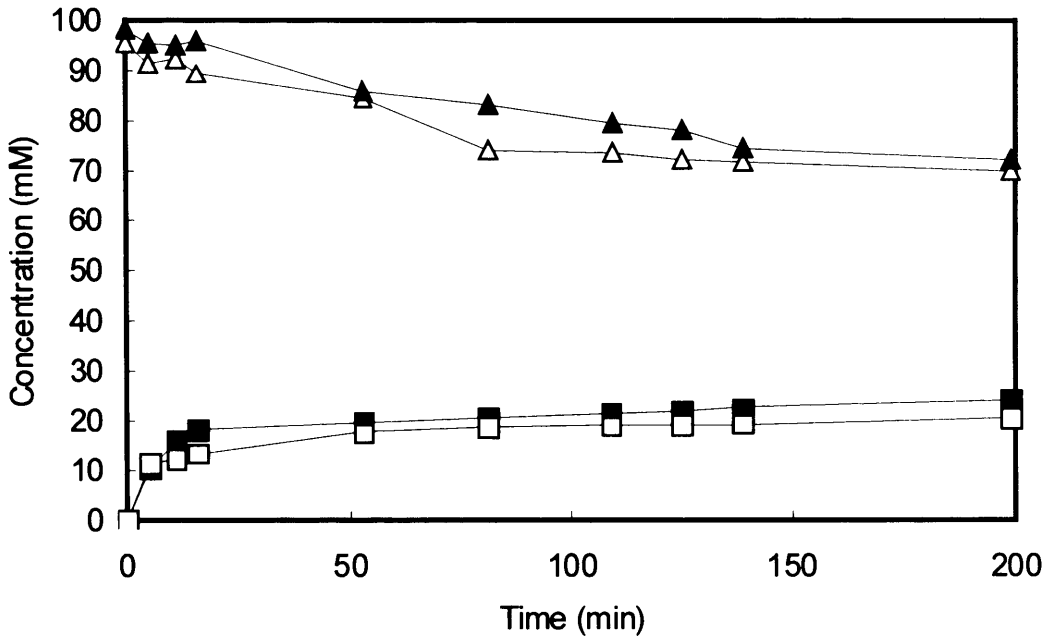


Figure 3.12 X5P preparation from 50 mM DHAP (-□-) in comparison to starting from 50 mM G3P (-■-) also indicating the levels of HPA present in the DHAP (-△-) and G3P (-▲-) reactions.

At increased concentrations (100mM) however there was a shifting of this behaviour, as the initial rate of G3P reactions was lower than those of DHAP. An example of this is shown in Figure 3.13. Initial rate of the DHAP reaction at 100

mM was 1.57 mM min^{-1} whilst the initial rate of the G3P was approximately 0.7 mM per minute. Interestingly at this high concentration the DHAP reaction experienced better yields and conversions. The gap between the two processes is reduced at this concentration only in terms of conversion.

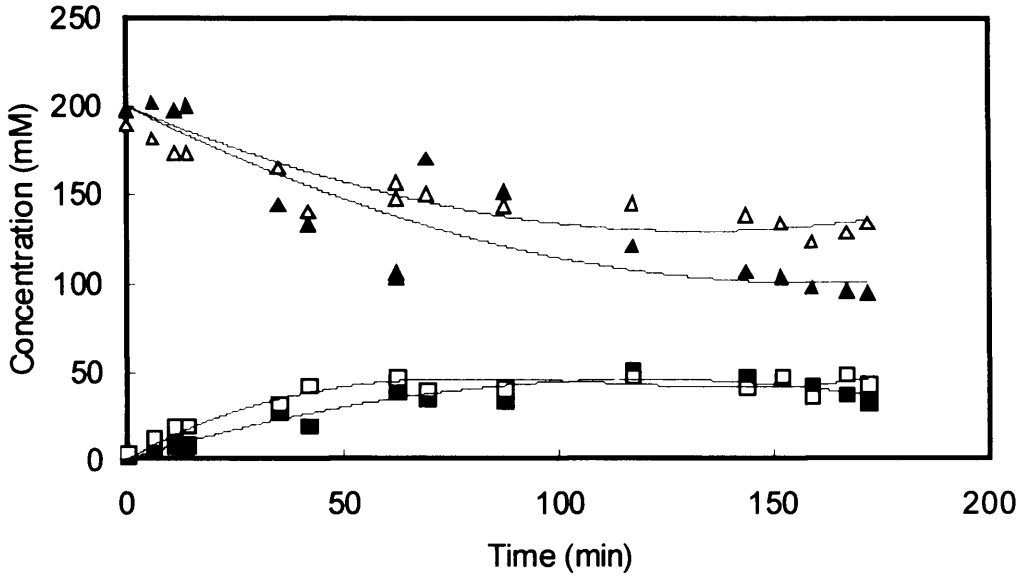


Figure 3.13 The resulting X5P from G3P (–■–) and DHAP (–□–) at 100 mM concentrations with decreasing HPA levels for the G3P (–▲–) and the DHAP (–△–) reaction.

The yield of X5P from substrate at 100mM levels was 48.5%. This value was true for reactions starting with DHAP and G3P. The conversion was shown to decrease with increasing substrate concentrations. The yield was lower for increasing amounts of substrate placed in the reactor but the concentration of product was higher. A final comparison of all the results was made by looking at the highest yields achieved at each concentration. These values were calculated in terms of X5P produced from substrate (mM). The results shown in Table 3.2 conveyed that as substrate concentration increases yield decreases. The difference of yield from G3P and DHAP also decreased with increasing concentration.

Substrate (G3P or DHAP) concentration (mM)	Yield [X5P]/ [G3P]	Yield [X5P]/[DHAP]
5	1.00	0.80
15	0.85	0.73
20	0.76	0.67
50	0.54	0.50
100	0.47	0.48

Table 3.2 Showing the effects of increasing substrate concentration (mM) on yield of product from substrate ($Y_{[P]/[S]}$).

As concentration impacted yield it was also observed that the initial rates of reaction were also concentration dependent. This was a definite sign of some degree of substrate inhibition. To clarify this the rates of X5P production in the above biotransformations were pooled together and an activity profile for the two substrates was constructed. To compare the G3P and DHAP activity profiles they were both plotted in Figure 3.14.

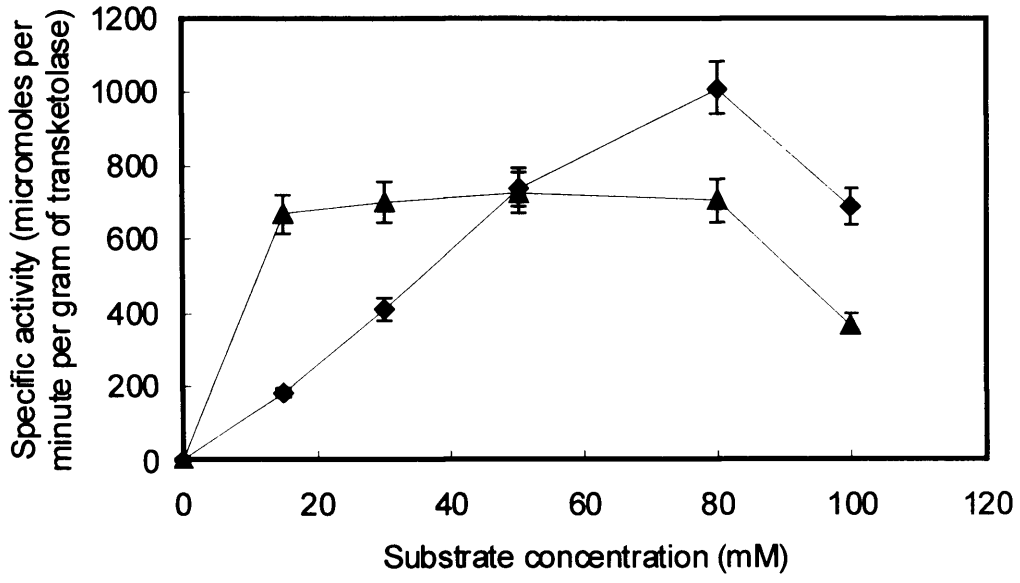


Figure 3.14 A comparison of initial activity profiles for the two substrates DHAP (—◆—) and G3P (—▲—) where specific activities were calculated based on initial activity μmoles $min^{-1}g^{-1}$ of transketolase.

The initial activities of the biotransformations were different depending on the starting substrate. When DHAP was used in a biotransformation the initial rates were slower than those of reactions starting with G3P. From Figure 3.14 it was observed that for concentrations below 50 mM the initial rates of reaction were lower in DHAP reactions than in G3P reactions. In the G3P reactions it was calculated that transketolase had a high affinity for G3P. The K_m was calculated from the results gathered above to be approximately 2 mM. This number corresponded with previously reported data (Sprenger *et al.*, 1995). The maximum rate achieved for the G3P reaction was on average 1.5 mM per minute. This activity was achievable in the substrate concentration range of 15mM-80mM. DHAP reactions on the other hand had lower initial reaction rates than G3P biotransformations when substrate concentrations were below 50 mM. This was presumed to be due to the reaction being limited by TPI converting DHAP to G3P. In the substrate concentration range of 50mM-80mM the DHAP reaction achieved higher initial rates. The highest rate achieved in a DHAP bioconversion was approximately 2 mM per minute. G3P conversions experienced reaction rates of 1.5 mM per minute at this stage. This lower rate was probably attributed some level of substrate inhibition. At substrate concentrations higher than 100 mM the initial production rates dropped. This was experienced in both DHAP and G3P biotransformations. These dropping rates were again possibly caused by an inhibition. To investigate this phenomenon further experiments were carried out to fully characterise the impact of substrates and product on the enzyme transketolase. These experiments were considered to be key in the next stage of characterising this model multi-enzymatic system. Testing for toxicity and inhibition is an important stage of characterising a biocatalytic system. The information was deemed necessary to distinguish why the rates of reactions and space time yield (STY) or productivity dropped at high concentrations of substrate.

Following the substrate stability experiments greater attention was given to running the process starting with G3P and DHAP rather that fructose 1,6 bisphosphate. This was due to the poor results obtained in Section 3.3.1. It was possible that the poor productivities were probably due to the long reaction timescale where the substrates could degrade and transketolase may lose activity. Based on the substrate experiments it was clear that the fructose 1,6 bisphosphate reaction suffered from instability of the components over the long time period. This reaction was seen to

suffer from low reaction rates and eventually require high energy input. These were the primary reasons for the hindrance of X5P production industrially so far.

Running the reactions from G3P and DHAP proved an important solution to these problems. By cutting out aldolase out of the reaction and starting from DHAP or G3P the time scale of the reactions was reduced dramatically. It was considered that the lag phase in the fructose 1,6 bisphosphate reaction was due to the inefficiency of the aldolase, probably due to the incorrect levels of enzymes present in the system.

By dissecting the reaction in this manner the characterisation of the system became a more manageable task. This was a major advantage where for the first time it was possible to investigate the direct involvement of HPA in the reaction. HPA is a key substrate not only as it is a rather valuable component but also that it has the major part in the completion of the reaction. The experiments in Section 3.51 showed that HPA must always be in excess of substrate to push the transketolase reaction forward. Figure 3.9 suggested that HPA concentration should be double to that of starting substrate for acceptable conversion of above 60%. In the methods previously described in literature this important feature was not adhered to and could explain the slow rates. The results also highlighted the importance of paying attention to the levels of components in the bioreactor at any given time during the reaction. The overall final concentration of HPA added to the reactions described in literature was approximately double to Fru1,6BP. However this addition took place over a long period of time (HPA was added at intervals). During the biotransformation the HPA levels in the bioreactor were very low and resulted in poor reaction attributes.

Using G3P as a starting substrate for the production of X5P proved very successful (Figure 3.10). This was to be expected as transketolase has a high affinity for G3P. The reactions carried out here were used to build a more detailed analysis of G3P as a substrate; Initial rates were calculated as well as yields. The results confirmed that for transketolase the Michaelis constant (Km) value was approximately 20 mM as previously indicated in literature (Zimmermann *et al.*, 1999 and Sprenger *et al.*, 1995). More importantly the results showed that at high G3P concentrations the rate of production decreases (Figure 3.14). The yield of [P]/[S] (yield on reactant) also decreased with increasing G3P concentration suggesting that the reaction was subject to substrate inhibition.

In the reactions utilising DHAP as a substrate the results were analysed with care, as TPI and Tk were both directly involved. It was necessary to consider the biocatalyst effect to fully understand the results. The substrates experiments showed lower yields in comparison to G3P but overall the results were very comparable (Figure 3.12).

Again at high concentrations the yields and rates of reaction dropped suggesting substrate inhibition. Product inhibition could be evaluated by a comparison of the initial rates and the yields. It was deemed necessary however to fully address the issue and identify the direct inhibitory/toxic effect of the substrates on transketolase. At this point guidelines were emerging on the levels of substrates necessary for the betterment of the process. This indicated the need for moving on to the next stage of experimentation (toxicity of the components).

3.3.4 Inhibition/Toxicity

The following experiments were devised to clarify what was taking place in the reaction vessel at high concentrations of substrate to reduce the reaction rates and yields. The experiments were focussed on identifying the impact of substrates and product on the key enzyme transketolase. The reaction components G3P, DHAP and X5P were individually incubated with a known constant amount of transketolase for 6 hours. The incubation took place at pH 7.0 and temperature of 25 °C. Transketolase activity was measured before and after incubation with each component.

3.3.4.1 Toxic effects of X5P on transketolase activity

Although it is often possible to evaluate product inhibition by comparison of the initial rates in this study this was not considered accurate. As there are a variety of components involved in a multi-enzymatic system it is important to examine their individual inhibitory effects. Pure product X5P was incubated with transketolase at varying concentrations for six hours at pH 7.0. The residual activity of transketolase was calculated before and after the incubation event. The percentages of remaining activities are shown in Figure 3.15. The results indicated that the presence of X5P did have a negative effect on transketolase activity. The maximum observed toxicity was 26% at 50 mM concentrations. It was difficult to obtain pure

X5P commercially. This was the reason for the small number of data points on Figure 3.15. The impact of this small group of data on the results accuracy was noted. However it was also important to carry out these important toxicity experiments to gain an overall understanding of product toxicity.

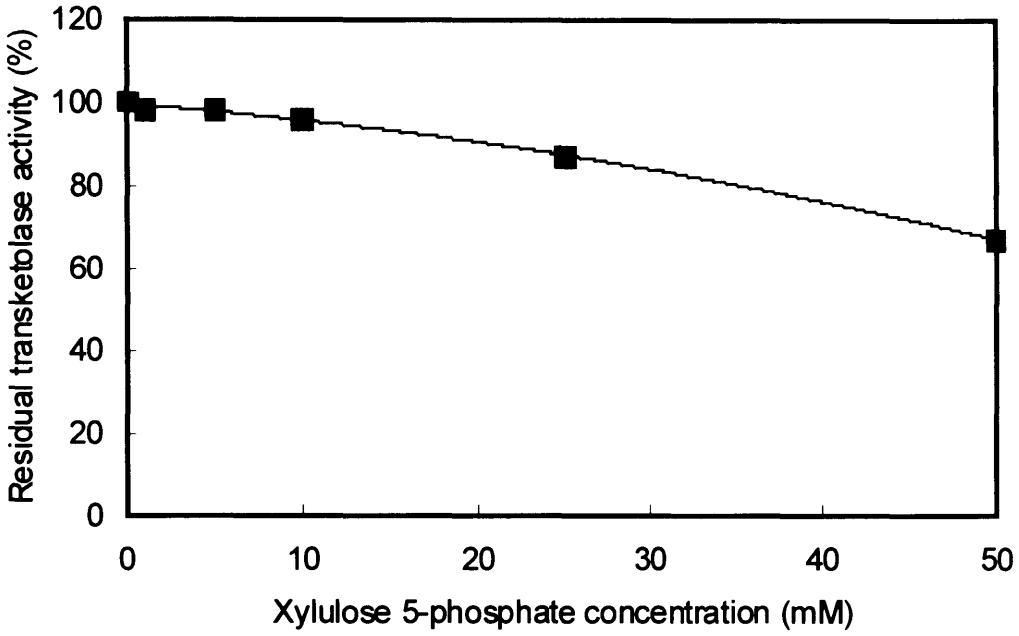


Figure 3.15 The toxicity of X5P (—■—) indicated by the residual transketolase activity (%) after 6 hours.

The results here suggested that the product concentration below 30mM had caused less than 20% damage on the transketolase. These data were considered highly important as they could be used to place limits on the final model process. This would depend on whether 20% loss of activity is deemed acceptable or not. It was apparent that at this early stage decisions could be made on the final process by carrying out these experiments.

3.3.4.2 Substrate toxicity

A number of experiments using the same procedure as above were carried out to identify the impact of G3P and DHAP on transketolase. The results are clearly described in Figure 3.16. The values for G3P and DHAP were plotted in this figure alongside each other to make a comparison. They were incubated with transketolase independently of each other.

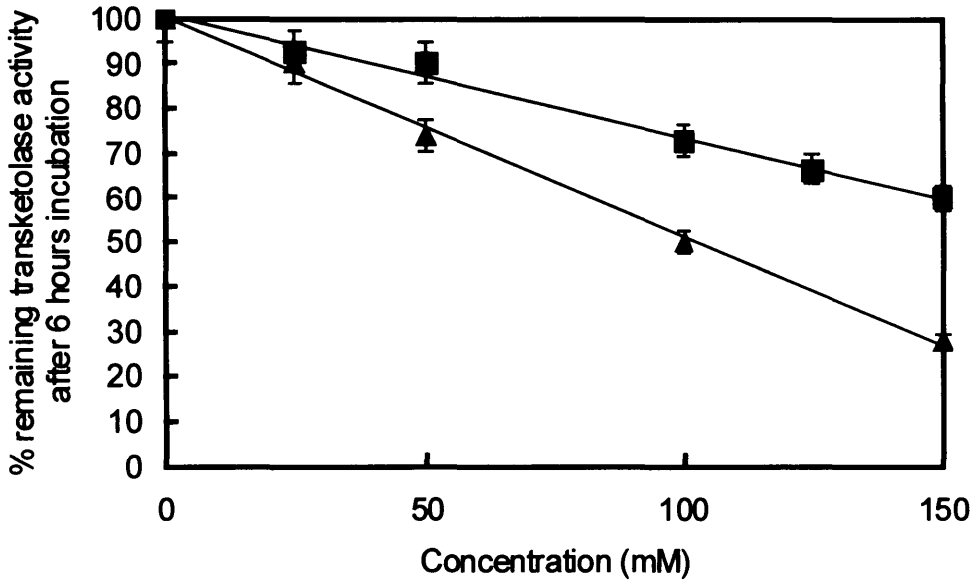


Figure 3.16 A comparison of substrate toxicity indicated by the remaining transketolase activity (%) after 6 hours of incubation with DHAP (—■—) and G3P (—▲—) at increasing concentrations. Error bars calculated from triplicate assay.

The results suggested that the substrates damage transketolase activity to a higher extent than the product X5P. It was indicated that G3P was significantly more toxic to X5P in comparison to DHAP particularly at concentrations higher than 80 mM. After the six hours of incubation the activity of transketolase was reduced significantly with G3P by around 70% at 150 mM concentrations.

It was not possible to remove G3P from the mixture. Consequently it was not possible to observe whether the activity returned to its original value after the incubation. The results did not fully explain the nature of the inhibitor. The results

obtained here do not provide detailed reasons for the inhibition. The values obtained were used to identify guidelines on the use of substrates. This reduction in transketolase activity was labeled the toxic effect. The toxic effect is an irreversible activity loss (Mitra *et al.*, 1998) and at this point the results did not offer any more explanation. To identify whether a component causes inhibition (competitive or non-competitive) it must first be removed form the reaction mixture. Then an activity test is carried out to identify whether the enzyme regains its activity (whether the inhibition is reversible). In the case of a toxic compound even with its removal the enzyme does not regain activity. To remove the components from the reaction mixture an in-situ product recovery ISPR technique needed to be implemented. This was beyond the scope of this thesis as whether the loss was due to an inhibitory or a toxic effect it was more important to identify the level of impact on the enzyme rather than it's nature. These experiments may be carried out at later stages of process development even after the final process is chosen. Designing a feeding strategy (fed-batch operation) or increasing biocatalyst levels are common solutions when faced with substrate inhibition / toxicity. As indicated by the results shown in Figures 3.15 and 3.16 transketolase is very sensitive to the components involved in the reaction. It was decided that a number of experiments needed to be carried out to fully explain the interplay of the biocatalysts and substrates involved in this multi-enzymatic model.

The experiments describing substrate and product toxicity indicated that G3P was certainly responsible for a greater loss of transketolase activity than DHAP. Due to low availability of X5P, its inhibitory effects on Tk were not fully analysed. X5P inhibitory effects are shown by the data in Figure 3.15. Although the effects might seem minimal (below 30%) at lab-scale they might prove to be detrimental in industry. The toxic properties of X5P were not further studied here. It is possible to develop an ISPR method for the removal X5P however this was not pursued during this research. The reason being that the concentrations of X5P produced so far in the literature were very low (below 20 mM). From the data gathered for Figure 3.15 it was shown that this concentration did not cause a major toxic/inhibitory effect on transketolase.

It was important to identify whether the components had an inhibitory effect or whether it could be classified as toxicity. The experiments used here to investigate this were rather simplified and only suffice as a quick indication. A quick

indication can prove vitally important when in the first stages of process development. To fully characterise whether the effects are toxic or inhibitory the components needed to be removed from the enzyme solution after incubation. The enzyme must then be tested again for its activity to identify the nature of the negative effect. In a reversible inhibition the transketolase will regain activity once the component is removed. This experiment was not carried out at this stage but in the final stages of process development it is necessary to gather as much information as possible about the nature of inhibition, competitive or non competitive caused by process components.

3.3.5 Levels of biocatalyst

The following experiments were devised to identify the role that the biocatalysts played in the multi-enzymatic synthesis of X5P. A series of biotransformations were conducted at small scale (5 mL). The reaction profiles were monitored for rising levels of product as well as decreasing levels of substrate. For simplicity the decreasing levels of HPA are not shown on the plots. It was considered important to observe biotransformations with increasing levels of TPI as this was thought to be limiting the DHAP reaction (Section 3.5). Increasing levels of transketolase were also of interest, as the G3P reaction kinetics was deemed dependent on this.

3.3.5.1 Effects of changing levels of TPI

It was highly important to gather data on the interplay of enzymes. This was considered to be of extreme importance when characterising a multi-enzymatic system. By increasing levels of TPI in the biotransformations it was possible to observe how production depended on TPI in the DHAP reactions.

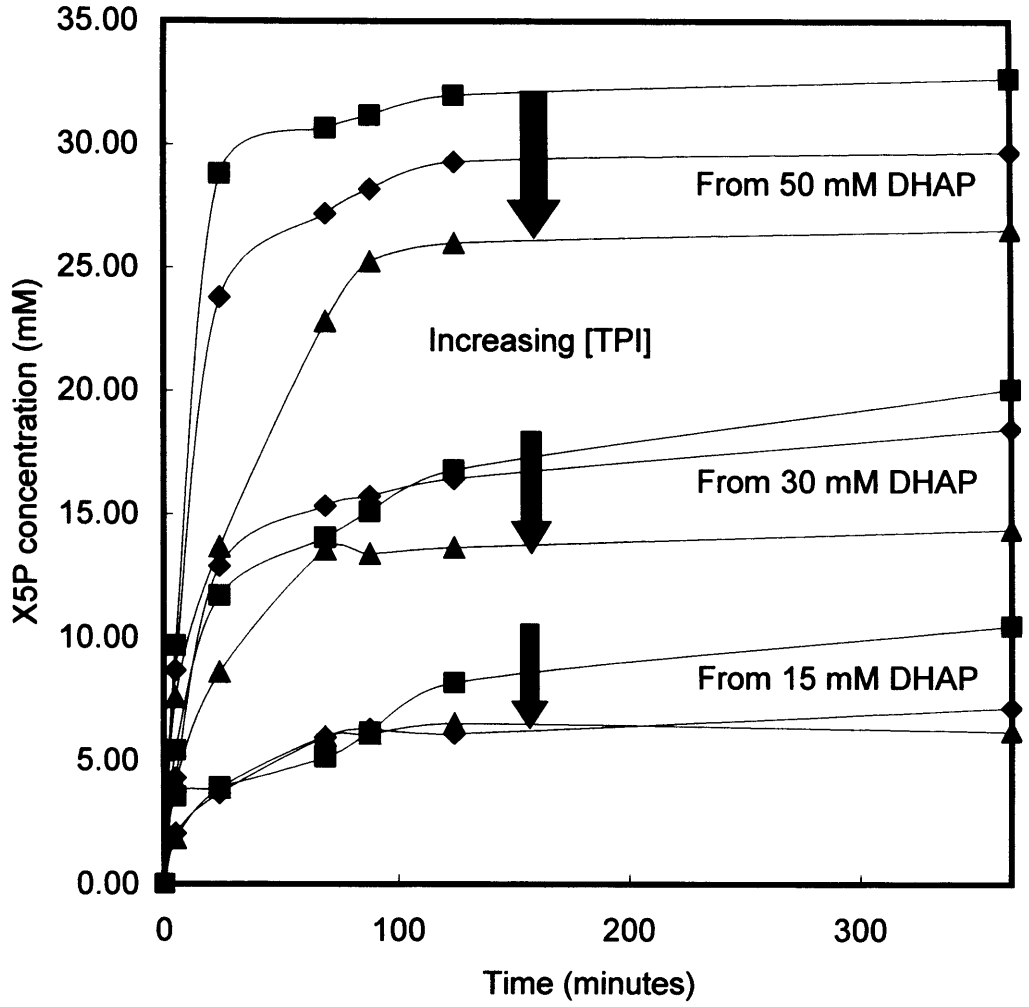


Figure 3.17 The preparation of X5P with increasing TPI levels in the reaction at 200 U mL^{-1} (—▲—), 40 U mL^{-1} (—◆—) and levels naturally present in Tki, 20 U mL^{-1} (—■—). The arrows show the increasing TPI concentration in reactions.

Pure TPI was added to transketolase and biotransformations were conducted with 15mM, 30mM and 50mM concentrations of DHAP. These biotransformations were run at low concentrations to eliminate the possibility of substrate inhibition as much as possible. The reaction profiles are shown collectively in Figure 3.17. The results show that increasing TPI concentration does impact the reaction kinetics and thermodynamic. By raising the TPI concentration in the biotransformations the ratio of TPI to Tk was in effect changed. At high levels of TPI the production

suffers as well as the initial rate of reaction. The yield of product based on substrate decreases from 65% to 53%. This was as a result of not enough DHAP being converted to X5P. It also illustrated that at high levels of TPI there was a lack of G3P in the system. Transketolase was probably not able to compete with high levels of TPI and the added competition for G3P. Increasing concentration magnified this effect. The results showed that when starting the reactions with DHAP the effects of the second enzyme (TPI) in the system must also be considered. Above 40 U mL^{-1} TPI the process falls below the efficiency expected. There is also a minimum limit to TPI as it is needed in converting DHAP to G3P.

3.3.5.2 Using increasing levels of transketolase

The same methodology was applied to investigate the levels of transketolase in the system. Biotransformations were conducted with increasing amounts of transketolase added. The level of TPI was kept constant in all reactions. Again this resulted in the change of TPI to Tk ratio. These experiments were carried out to identify the best levels of enzyme to use in a X5P synthesis. Using this method of changing ratios it was possible to identify the optimal enzymatic levels for a multi-enzymatic system. The results suggested that transketolase also hindered the reaction at high levels. The reaction profiles for increasing transketolase levels are shown in Figure 3.18.

Crude transketolase as prepared using the methods in this thesis contained TPI as a fraction of the intracellular protein. TPI level as a ratio to Tk was shown to be rather high in terms of activity (approximately 40:1). It was considered that by adding pure transketolase to biotransformations the ratio of TPI to Tk could be changed. It was also important to therefore understand the impact of increasing transketolase levels on X5P preparation. Adding increasing amounts of pure transketolase to the biotransformations allowed this. The results show a significant decrease in the resulting X5P as levels of transketolase increase in the reaction mixture. At high levels of transketolase not enough G3P was being converted to X5P. It was considered that high levels of transketolase also had an effect on the TPI reaction in converting DHAP to G3P. The equilibrium was perhaps pushed towards DHAP. The production did not reach it's potential.

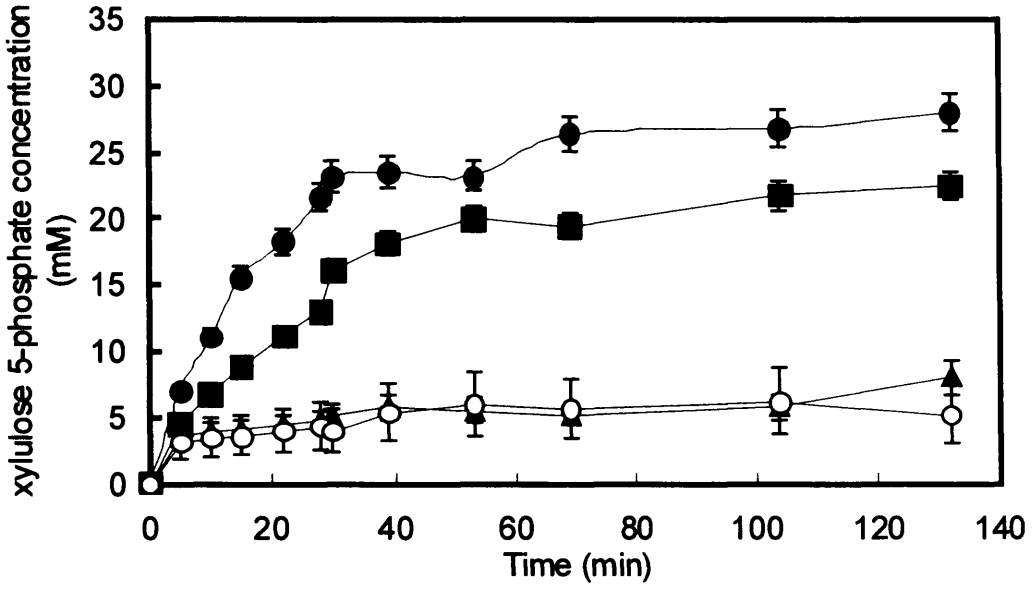


Figure 3.18 The preparation of X5P with increasing transketolase levels at 1 U mL^{-1} (—●—), 4 U mL^{-1} (—■—), 8 U mL^{-1} (—▲—) and 12 U mL^{-1} (—○—). Error bars are calculated from triplicate sample analysis.

Certainly the most intricate stage of the characterisation was identifying the interplay of the biocatalysts. An in-depth study of this issue was found be very time consuming. It was proposed here to carry out two integral investigations:

1) The effects of increasing levels of [TPI] keeping Tk constant.
2) The effects of increasing [Tk] keeping TPI levels constant.

If more enzymes are involved in the system the number of investigations increases accordingly. This enabled the calculation of the optimal enzyme ratio (TPI/Tk). The investigations may also be tested in a variety of starting substrate levels to identify how concentration comes into play with enzyme ratio. The results suggested that as TPI levels increased with respect to Tk the yield and the rates of reaction decreased (Figure 3.17). It was possible to explain this effect as both TPI and Tk compete for the same substrate pool of G3P. At high levels of TPI the G3P

was more quickly transformed back to DHAP. Consequently there was a lack of free G3P in the system or similarly transketolase could not compete with the high levels of TPI.

When increasing transketolase levels in the system a similar effect was observed as yield and rates of reaction decreased. It is expected that at high levels of transketolase there was not enough free G3P available for the reaction to reach completion. The G3P level had fallen below the Michaelis constant (Km) value for the reaction. The high concentration of transketolase may have had a direct effect on the TPI reaction and may have pushed the equilibrium more towards DHAP. It may have even halted the TPI reaction completely. This meant that DHAP is not converted to G3P and the reaction did not reach completion.

3.3.6 The logic of characterising a multi-enzymatic system

When faced with an unknown complex reaction and its characterisation, it is necessary to collect only the data required for the definition of the constraints. This reduces the time needed to develop the process for industrial use. To successfully implement this a list of key experiments must be carried out (Mitra *et al.*, 1998).

The priority given to these experiments is dependant on a range of issues, which come to the foreground when envisaging the scale-up or the industrial use of the system. These issues include process efficiency, bioreactor design, bioreactor cost, product cost, substrate cost and biocatalyst cost. Process efficiency is addressed in terms of yield, product concentration and productivity for the different classes of target compounds (Straathof *et al.*, 2002).

Based on what is the most important issue the experiments were geared towards that key aspect. This meant that the experimental route taken by the biochemical engineer changed based on the key aspects of the system. A profitable process is one in which the resulting product is more valuable than the raw materials involved (Rudd, Powers and Siirola, 1973).

Assuming that the product is more valuable than the cost of the raw materials (enzymes and substrates) it was possible to concentrate on solving issues such as the levels of substrate to use and the levels of enzymes to use. In the case of low value enzymes and high value substrates it is imperative to give priority to

experiments identifying the substrate stability and the levels of substrates to be used in the system.

In the opposite scenario where the enzymes are more valuable and the substrates are more readily available (less valuable) other experiments come to the fore ground such as enzyme purity, toxicity / inhibition and amounts of enzyme needed for the reaction. Figure 3.19 shows the proposed logical route of experiments that must be carried out to successfully characterise a multi-enzymatic system. By putting this logic into action the characterisation of any given system is possible in the shortest time and the research becomes more focused and efficient. Carrying out key experiments cuts down the development time. Also this is imperative in research where often only minute amounts of substances are available to characterise systems.

In the production of X5P the enzyme transketolase has been over-expressed in *E. coli* and produced in large fed-batch fermentations with very high yields. This enzyme is considered to be readily available and sourced cheaply. The enzyme TPI is also readily available in high quantities for low prices. Here the substrates involved, HPA, DHAP and in particular G3P are considered the most valuable feature in the system. Therefore based on this assumption the characterisation experiments were geared towards solving the substrate issue first (Route 2 in Figure 3.19).

Following route 2 in Figure 3.19 meant that first the substrates were compared based on stability. It was considered that monitoring levels of substrate stability was an important first stage towards the characterisation of this model system. It was deemed reasonable to investigate other aspects of the substrates such as their volatility and solubility. This depends on the model process. These experiments were not achieved during researching this model system. Information was readily available on the substances as well as the biocatalyst in literature. Additional substrate experiments may need to be carried out when applying this characterisation methodology to other systems. A component might be involved that is highly hydrophobic or highly volatile. A substrate may suffer from poor solubility. These all differ on a case-to-case basis. What is important is in the manner in which the experiments are approached. By following route 2 if Figure 3.19 a decision was made to investigate the substrate properties first. In particular substrate stability as it was most relevant to the model reaction.

There have been many other routes suggested in literature for the characterisation of enzymatic systems. Blayer and co-workers put an example of one of these strategies forward. The rationale was to make early process decisions based on a minimum number of experiments (Blayer *et al.*, 1996).

The experimentation was divided into two sets. The first being component characterisation, concerning reactants and products. In this stage important information was gathered from literature and experimentally on the reactants, product and the biocatalyst. The second sets of experiments were in aid of interaction characterisation including stability, kinetic parameters, reactant (s) and product (s) toxicity. The two sets of data guided the engineer to process choices as rapidly as possible with a high degree of confidence (Blayer *et al.*, 1996).

The work here and the strategy proposed by this chapter extrapolated on the philosophy of characterisation found in literature. This was considered necessary as reactions in biocatalysis become more complicated and intricate. In a multi-enzymatic synthesis it is not possible to characterise the components independently of the interactions. The experiments must rationally move between these two types of characterisation. This is important if process parameters are to be defined as quickly as possible.

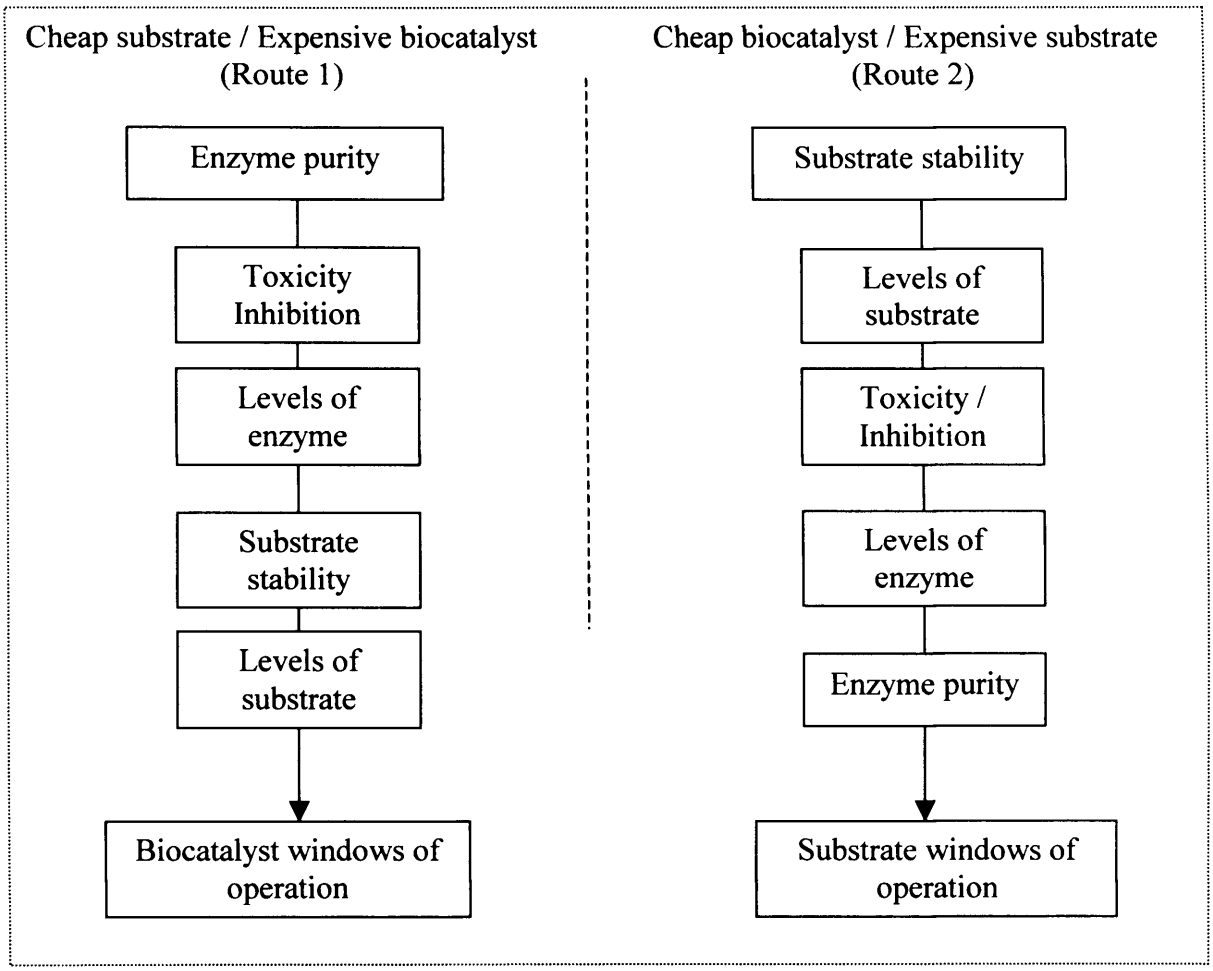


Figure 3.19 The logic of multi-enzymatic characterization.

The route of what parameters must be identified and considered is dependent on the value of the substrates and the enzymes assuming that the product is more valuable than all. The routes shown above (Figure 3.19) were shown by the results to be fully applicable to the multi-enzymatic synthesis. It was noted that changing routes was easily possible as more data was gathered. The results also highlighted the importance of knowing when to move on to the next stage. This was a difficult decision and one that often slowed down the time of process development. The results here conveyed that at each stage there was a distinct point when enough data was available to place process parameters or limits. This was thought to be the point at which the scientist must move on to the next experimental stage.

3.3.7 Thermodynamics and kinetics

It was clear from the results that there are two main methods for manipulating a multi-enzymatic system to achieve a better yield or productivity.

1- By compromising the relative levels of substrates in the system. Focusing on the yield based on substrate and the equilibrium.
2- By compromising the levels of enzymes in the system. Focusing on the kinetics.

However it was apparent that it would not suffice to take on board only one of these methods. These two methods are not independent of each other. Substrate concentration was shown to have a direct impact on the kinetics and the thermodynamics of the enzyme ratio and vice versa (Figure 3.18). As experienced here when dealing with the substrates levels after a certain point the involvement of the enzymes shows its importance. When investigating the ratios of enzymes to use the importance of the substrates levels starts to become apparent. It was therefore was necessary to move back and forth between issues to get a fuller picture of how the system really works. This means that a loop occurs in the experimentation between thermodynamics and kinetics of the system. Figure 3.20 explains this theory further.

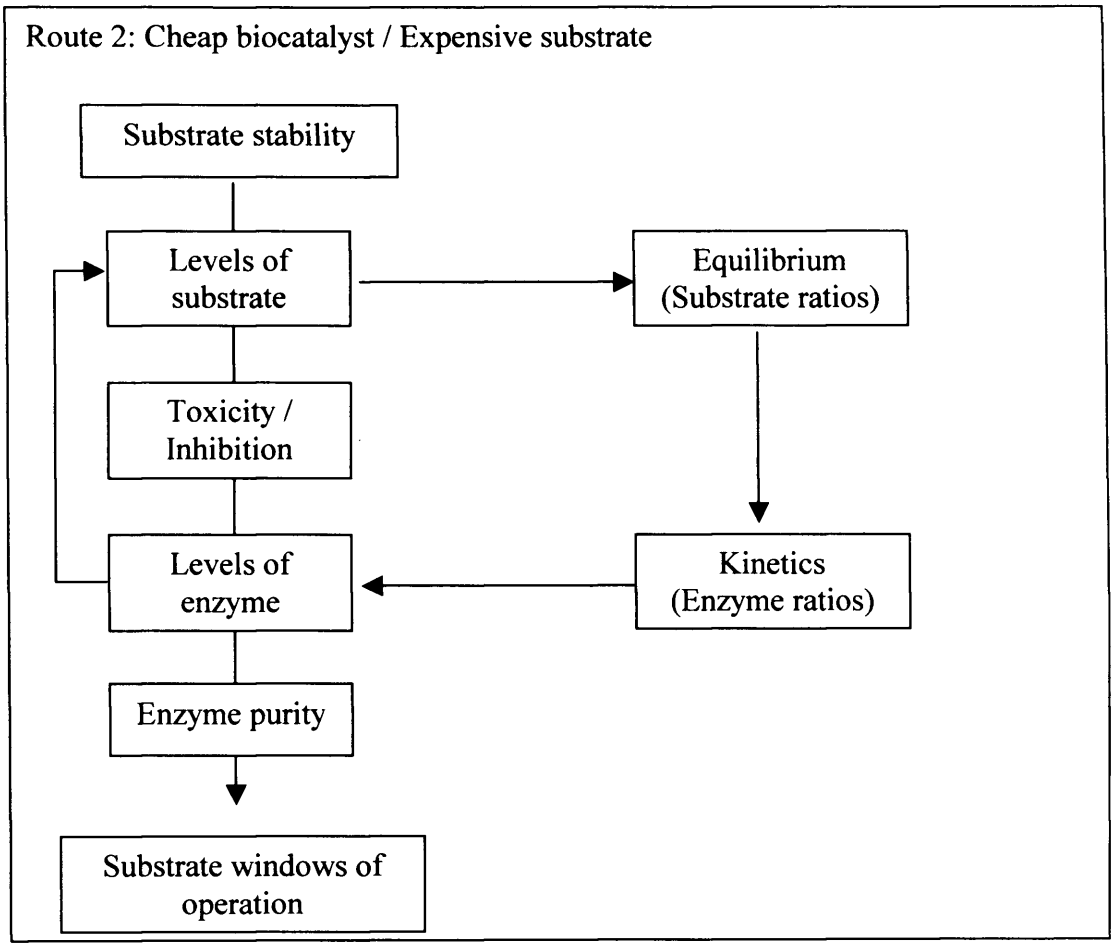


Figure 3.20 The impact of Equilibrium and kinetics on the route taken for the characterization of a multi-enzymatic system.

3.4 Discussion

The work in this chapter showed the characterisation of a model multi-enzymatic synthetic reaction in order to define the constraints for process selection and scaleup. Firstly the reaction was considered as a one-pot system starting with fructose 1,6 bisphosphate and HPA as substrates was conducted. The results showed that the reaction was slow and the time scale meant that further transketolase and HPA needed to be added. The inclusion of the cofactor TPP increases the susceptibility of the enzyme to oxidative attack. The loss of transketolase activity may have been due to this as well as the presence of the substrates, which may have caused a toxic effect. The results confirmed previous findings in literature. The multi-enzymatic system was dissected in order to investigate each aspect of the process. Thus it was possible to characterise the reactions in a logical manner. The progress towards meeting the aims of this chapter are discussed here.

3.4.1 Identifying the bottleneck

The natural progression was therefore to distinguish the key issues in a multi-enzymatic system. The results in this chapter indicated that G3P is the bottleneck to the whole reaction. The supply of G3P determined the final yield, affected the thermodynamics and the kinetics of the system. From the results it was clear that G3P supply directly affects the multi-enzymatic production of X5P. The reasons identifying G3P supply as the main bottleneck on the system were:

1- G3P is the most unstable component in the reaction. It loses its phosphate group in solution very quickly.
2- G3P is the most valuable component and only available commercially in small quantities.
3- G3Ps higher toxicity than the other components affects transketolase and renders the reaction more susceptible to enzyme denaturation.
4- G3P is converted to X5P in the presence of HPA. This gives the best yield (product / substrate).

5- DHAP must be first converted to G3P by TPI. The rate of this limits the overall reaction. Specifically the levels of G3P present in the reaction limit it.
6- G3P At high levels and in the presence of TPI drives the equilibrium towards DHAP. Directly effecting the X5P production.
7- G3P supply determines the kinetics and the thermodynamics of the system,

Identifying G3P as the bottleneck is an important step in characterising the multi-enzymatic X5P production. Manipulating its supply meant not only compromising enzyme levels but also compromising substrate concentrations. Thus stringing together the thermodynamics and kinetics issues. The results suggested that identifying the bottleneck is a major milestone in characterising a multi-enzymatic system. Significantly this provides the means to manipulate the system. Once the bottleneck has been identified the system is easily influenced either directly by changing the levels of that component or indirectly by changing other components that have an impact on the key component. Using the X5P system as an example it was possible to change the available level of G3P directly by changing substrate concentrations, or indirectly and perhaps more effectively by compromising the enzyme ratio. By gathering the results together it was possible to learn how to manipulate the system and draw some preliminary operational guidelines on the model process.

3.4.2 Operational guidelines based on results

Based on the findings in the results decisions can be made to place guidelines on process operation for a reaction involving more than one enzyme. Transketolase is a key enzyme in this synthesis and so the availability of G3P has been identified as the bottleneck to the production. Furthermore, it was described in the biotransformations here how sensitive the interplay and the interaction of the enzymes and substrates are. From these key sets of experiments it was possible to understand the circumstances involved in running a particular multi-enzymatic process. The best conditions for the educts involved have been identified by

stability studies. What impacts those components have on the enzyme and reaction productivity has been established. Subsequently the enzymatic interplay and substrate compromise was described.

The values that were found in these experiments can be presented in the form of operating windows of the kind suggested by Woodley and Titchener-Hooker (1996) for design of bioprocesses. For simplicity the production process was divided into sections. Firstly those starting from G3P and secondly those starting from DHAP. A comparison of these two scenarios was best achieved using the principle of windows of operation (Woodley and Titchener-Hooker, 1996).

The assumption was that the process must be economically sound and therefore cannot suffer losses greater than 40% whether it be due to substrate instability, enzyme denaturation or process inefficiency. A loss of 50% may be acceptable when dealing with a cheaply sourced raw material. Although these limits might change on a case-to-case basis the overall approach to placing guidelines (the shape of the process windows) is not altered. It is important to consider the axis of the windows of operation and decide what factors they should be defined by.

It was possible to use the characterization data gathered here to compare two processes. Those starting with G3P and those starting with DHAP. Analysing these two processes was predicted to inevitably provide vital information on how to manipulate the overall system too. Table 3.3 was used to simplify and clarify what information was gathered by the characterisation experiments. It was shown that constructing a table of the parameters and guidelines found during experimentation made the analysis of results simpler. This table was very useful in highlighting what was learned in the experiments and what impact the information gathered had on process design.

Data Gathered on:	Toxicity	Stability	Kinetics	Thermodynamics	TPI vs. TK ratio
G3P reactions	[G3P] at which 40 % toxic to biocatalyst	[G3P] Vs. pH	[G3P] Vs. Initial rate or [P]/[E]	[G3P] Vs. [P]/[S]	Not applicable
DHAP reactions	[DHAP] at which 40% toxic to biocatalyst	[DHAP] Vs. pH	[DHAP] Vs. Initial rate or [P]/[E]	[DHAP] Vs. [P]/[S]	[TPI]/[Tk} Vs. [P]/[S]

Table 3.3 Summary of the knowledge gained by the characterization experiments on each starting substrate.

Using the table above it was possible to group attributes that can be viewed together and form the boundaries of the windows i.e. toxicity and stability. It is notable that the accepted value for loss due to instability and toxicity was chosen as 40%. This value is subject to change. Changing this value has a direct effect on the size of the operable window for the process but not necessarily the shape. Being able to change limits is one of the advantages to drawing these maps. Flexibility is very important in process development. If boundaries should change the analysis is flexible enough to take new changes into consideration and still prove relevant. With regards to the reaction starting with G3P as a starting substrate. It was gathered that G3P is highly valuable and susceptible to pH changes. It was also the key bottleneck in the reaction. As a result its degradation in the bioreactor was regarded unacceptable from an engineering point of view. Analysing the table above it was possible to group stability and toxicity together with a common axis of pH and substrate concentration. The variables were plotted in Figure 3.21 with pH defining the area in which G3P and DHAP are unstable and fall below expectations at more than 40% loss. Based on the results it was apparent that the other important factor to consider in the process was G3P concentration (mM). From the data collected in the toxicity experiments it is apparent that at above 80mM levels G3P has detrimental effects on transketolase activity More than 40% loss. Hence there will be a limit to running processes higher than this concentration unless a feeding strategy is adopted. The plot in Figure 3.21 suggests that the operation

window for running a process starting with G3P is more restricted than that of one starting with DHAP. The toxic effect of DHAP was only experienced at concentrations higher than 150 mM. Moving this post from 40% to 20% will restrict both windows. It is possible to move these posts/guidelines at a later stage. The preliminary analysis of the DHAP and G3P processes shown in Figure 3.21 suggested operating guidelines related to the mode of operation (batch of fed-batch) and the physical reaction conditions such as substrate concentration and pH. It is apparent that in both cases the lower limit of pH is defined by the optimal activity of Tk at pH 7.0. This meant that a multi-enzymatic process for X5P production must be conducted with pH control and maintained at pH 7.0. Toxicity constraints also meant that for a batch system the concentration of the starting substrate must be below 150 mM for DHAP and 80 mM for G3P. If higher concentrations are to be used a feeding strategy must be designed for a fed batch system.

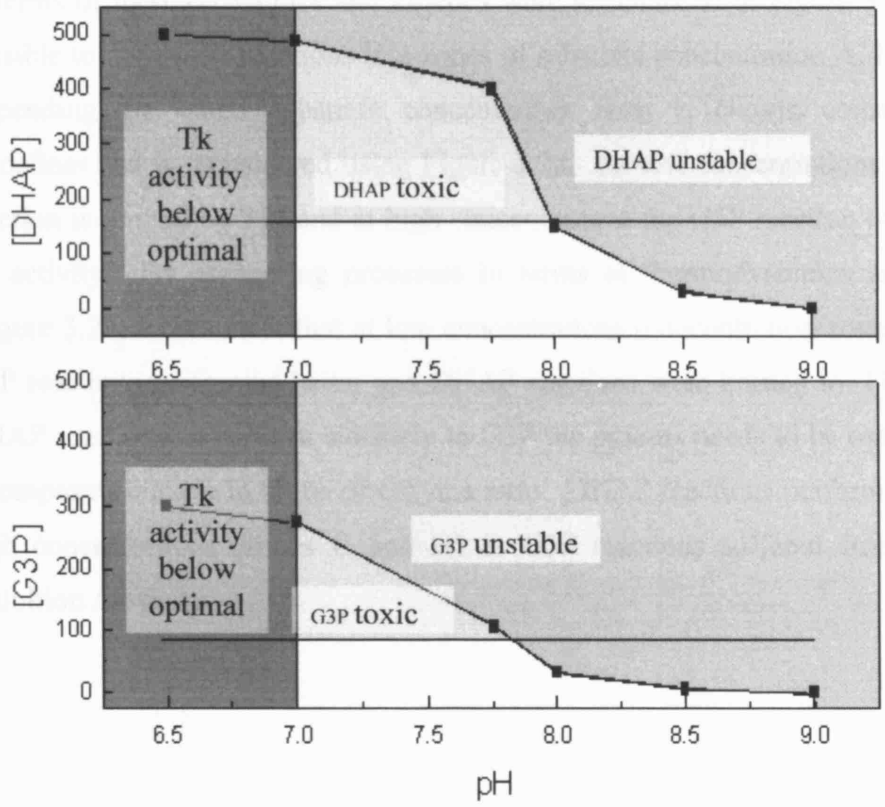


Figure 3.21 The operation windows for preparation of X5P starting with G3P and DHAP considering pH stability, substrate concentration, substrate stability and inhibition.

3.4.2.1 Operational guidelines based on kinetics and thermodynamics

Using the guidelines in Figure 3.21 a picture of the process at larger scale was beginning to form. It was important to consider other attributes of the system. From table 3.3 the issues of kinetics, thermodynamics and [TPI]/[Tk] ratio were yet to be addressed fully. To identify the operational windows considering these aspects first the results needed to be analysed in greater detail. Having defined the constraints of the bioreactor it was possible to use the results to delve deeper into

the windows and define the working conditions within them. In effect it was possible to identify windows of operation within windows of operation. The results in terms of thermodynamics and kinetics were summarised in Figure 3.22. It was possible to divide the reactions into zones of substrate concentration A, B, C and D. Depending on which substrate concentration zone is chosen constraints and guidelines can be considered using Figure 3.22. At low concentrations the DHAP reaction is limited by TPI and at high concentrations the G3P reaction is limited by Tk activity. By comparing processes in terms of thermodynamics and kinetics (Figure 3.23) it was clear that at low concentrations (concentration zones A and B) G3P reactions performed better and DHAP reactions were limited by TPI. For the DHAP reactions to perform similarly to G3P the process needs to be optimised and a compromise made in terms of enzyme ratio. DHAP reactions performed better at high concentrations (zones C and D) as G3P reactions suffered from substrate inhibition / toxicity.

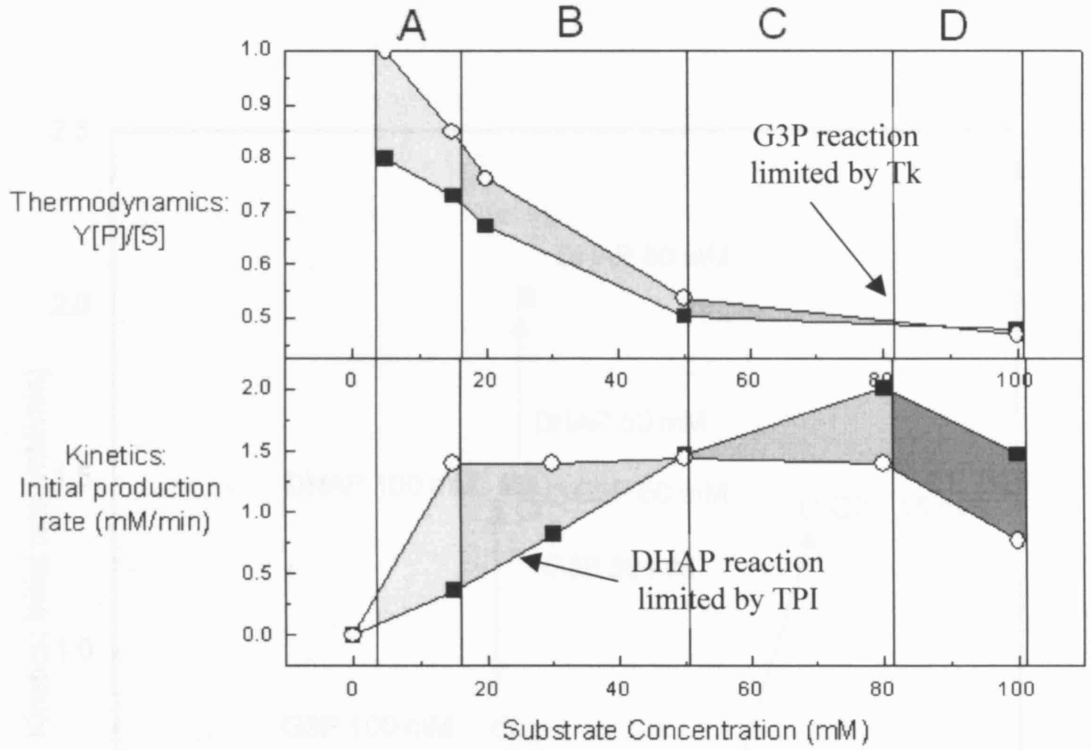


Figure 3.22 The kinetics and thermodynamics of reactions with increasing concentrations of G3P (—○—) and DHAP (—■—).

The arrows in Figure 3.23 indicate how much processes have to be optimised to compete with their counterparts in terms of kinetics and thermodynamics. At 15mM concentrations the DHAP reaction has to be improved using compromised biocatalyst levels to compete with the G3P reactions at the same concentration. At 100 mM concentrations however, the G3P reaction suffers drastically and it needs to be improved to compete with DHAP reactions at the same concentration. The only instance when the processes are very similar to each other in terms of kinetics and thermodynamics is at the 50 mM concentration.

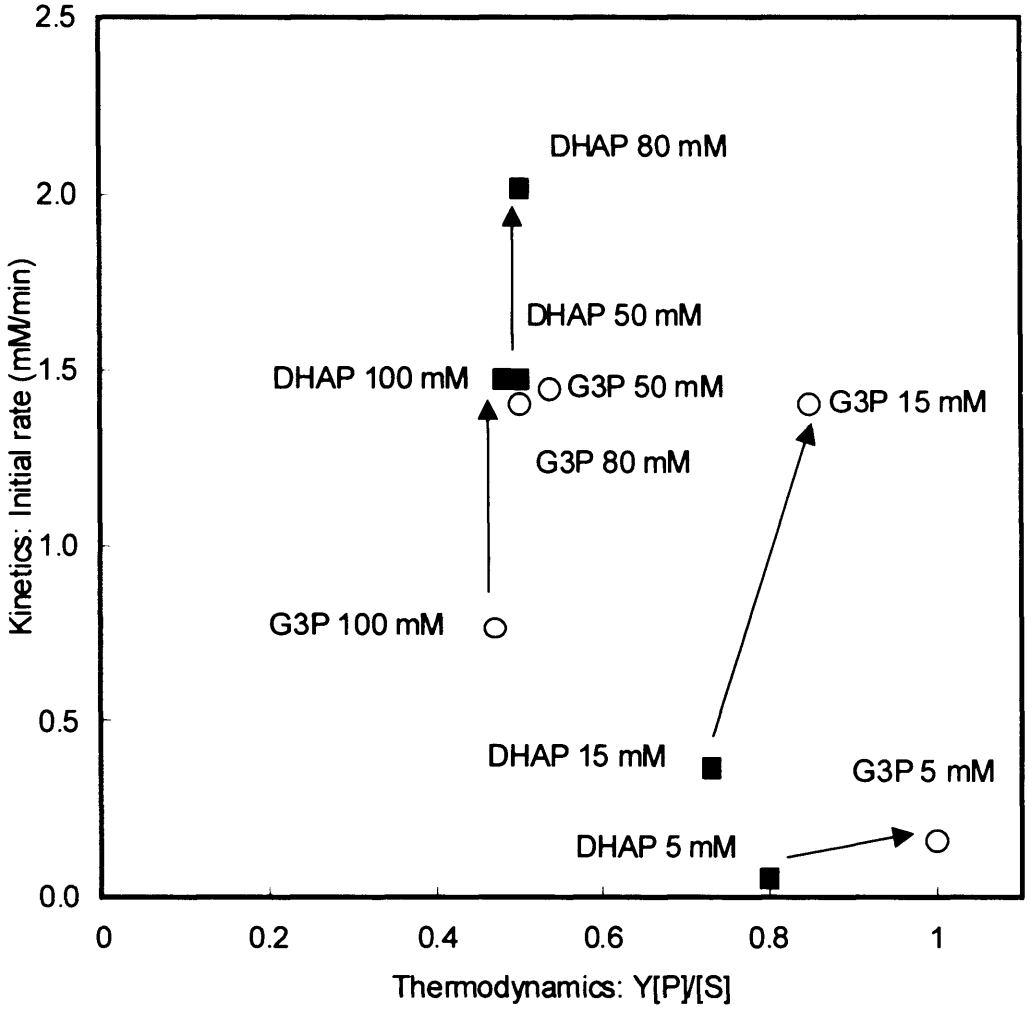


Figure 3.23 Process attributes in terms of thermodynamics and kinetics when operating with G3P (—○—) or DHAP (—■—) as starting substrate. Arrows show the difference between processes and how much one process has to improve to compete with the better design. The length of arrow represents the distance to optimization.

3.4.2.2 Further operational guidelines for the DHAP reaction

Using the data from Figure 3.17 it was possible to set an acceptable return on substrate (Yield) as 60%. A return of X5P lower than 60% was labelled as unacceptable thermodynamics. Based on this requirement at low concentrations of DHAP the TPI to Tk ratio had to be kept to the minimum. At higher concentrations of DHAP the yield on substrate was above 60% even at higher [TPI]/[Tk] ratios. These guidelines were used to construct a separate window of operation for DHAP reactions (Figure 3.24). This operational requirement of 60% $Y_{[P]/[S]}$ may be lowered to 50% or 40% depending on the perceived value of substrate. This action will widen the operable window.

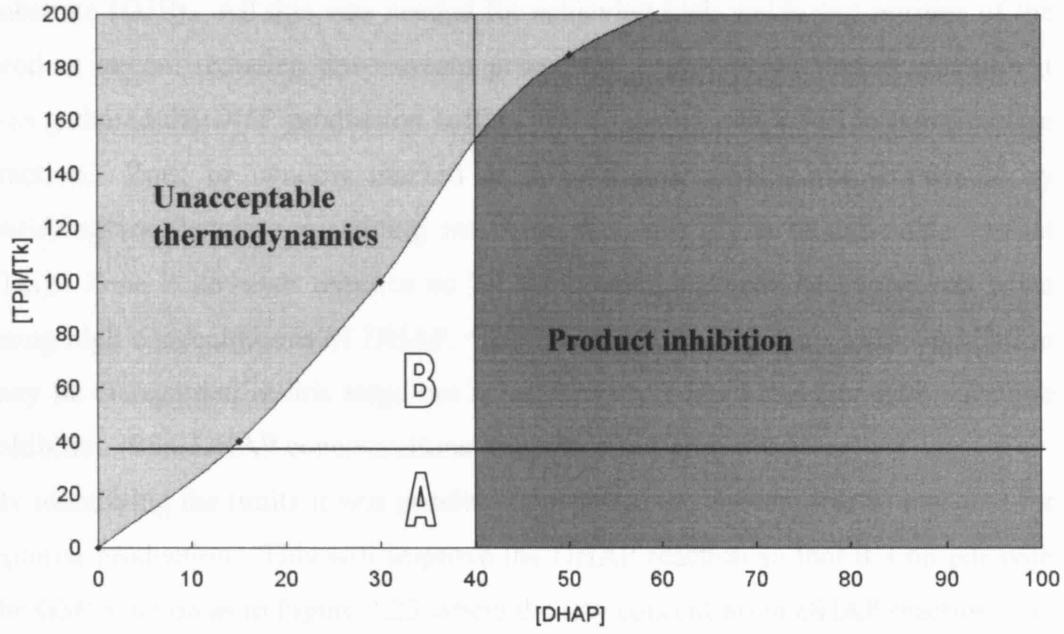


Figure 3.24 Operation window representing guidelines for running the DHAP reaction in terms of concentration and TPI/Tk compromise. Zone A shows the possible process window using below 40 [TPI]/[Tk] ratio. Zone B illustrates the possible operable window by increasing the enzyme ratio.

Figure 3.24 illustrated that the ratio of TPI to Tk was sensitive to DHAP concentration. The preparation of X5P from low concentrations of DHAP would require the smallest [TPI]/[Tk] ratio to achieve acceptable yields achievable by adding further Tkp to the crude extract and compromising the enzyme ratios. It can be argued that TPI level is also an issue when using semi-purified transketolase in the G3P reaction. The reaction does not go to completion unless pure transketolase is used. There was always some G3P converted back to DHAP. This was due to the crude transketolase extract containing TPI. There was approximately 20% difference in the $Y_{[P]/[S]}$ when comparing a reaction running with Tkp to one containing residual cellular TPI. At this stage a question of economics also comes to the foreground. The presence of TPI effects the reaction but the cost of Tkp can justify this hindrance caused by using a cheap crude extract. Or on the other hand using a pure enzyme is the only answer when employing an expensive, sensitive substrate (G3P). All this was needed for achieving high yields and purities of the product stream reducing downstream processing costs. From experimentation it was gathered that X5P production suffers in the case of a high TPI to transketolase fraction. Zone or window marked as A in Figure 3.24 is one achievable by purifying transketolase or adding more transketolase (Tkp) to the crude extract (Tki). Zone B however requires no Tk purification and may be considered when using high concentrations of DHAP. The boundary marking the product inhibition may be disregarded at this stage but it must be considered together with substrate inhibition if the DHAP concentrations increase in scale-up.

By identifying the limits it was possible to estimate the enzyme fraction needed for optimal production. This will improve the DHAP reaction so that it's on par with the G3P reaction as in Figure 3.23 where the low concentration DHAP reactions can be optimised to move near corresponding G3P reaction. The characterization data gathered on the multi-enzymatic system will be subsequently used to run the system at laboratory scale.

3.5 Summary

As stated in Section 3.1, the aim of this chapter was to characterise the X5P production. Characterisation of this model multi-enzymatic synthesis has provided

constraints and important clues as a basis for a rational approach to eventual process selection. In summary the approach taken here has shown the manner in which a multi-enzymatic reaction should be investigated. Providing a set of parameters to work with and identifying at an early stage the operating limits. The subsequent stage of process synthesis is one that relies largely on the guidelines that were drawn. Identifying the best process relies heavily on these data. The main results from this chapter are summarised below:

The substrates were affected by changing pH. The incubation of substrates and product over a range of pH values in the presence of cofactors indicated that all components were stable in acid but showed a marked instability under alkaline conditions at 25 °C. A comparison of G3P and DHAP showed that G3P was more sensitive to an increase in pH. DHAP was found to be more stable even at high pH values. The instability of the components was particularly evident at high initial concentrations but observed to a lesser extent at lower initial concentrations. Experiments on the reaction components indicated a pH limit on the final process.

The product X5P was shown to cause a toxic effect of greater than 20% above 30mM concentrations. This placed a possible limit on the subsequent processes. The substrates were inhibitory to the process. In particular G3P was shown to cause inhibition above acceptable levels at concentrations higher than 80mM. This suggested the need to introduce a feeding strategy to the system in subsequent processes. The levels of substrate had a notable impact on the reaction attributes.

It was highlighted by the results that the concentrations of substrates could be manipulated to improve the reaction yield, productivity and kinetics.

The results have shown effective production of X5P from the intermediates G3P and DHAP without the involvement of Fructose 1,6 bisphosphate or aldolase.

The levels of biocatalyst were shown to be of major importance in the reactions where TPI to Tk ratio in terms of activity directly affected the process yields and productivities.

The results showed clearly that the concentration of G3P in all reactions was of major importance and the bottleneck to the process.

As a result of this characterisation approach, operating windows were defined, relating the process boundaries in the form of diagrams.

This characterisation strategy is a valuable tool for subsequent scale-up. Based on this study at this early stage of process development some options have been eliminated. Possible processes that have been eliminated so far due to unfavourable attributes are:

1. Processes starting with Fru 1,6 BP as a starting substrate
2. High Concentration G3P batch processes (above 80 mM)
3. High concentration DHAP batch processes (above 150 mM)
4. Low concentration DHAP processes (below 5 mM)
5. DHAP processes with TPI/Tk above 40:1
6. Processes with low levels of HPA (ratio to starting substrate must be 2:1 on a molar basis)
7. Processes without pH control (product degradation at high pH)

These strategies can be applied to other pathways and provide the advantage of gathering data in a short space of time and aiding rapid process development. These findings are sufficient to identify the key constraints on the design of a bioconversion process for this model reaction. They also demonstrate the usefulness of adopting a systematic approach to characterisation. Having characterised the system the next step is to understand the design of a laboratory scale process and the importance of biocatalyst purity.

4 Laboratory scale preparation of xylulose 5-phosphate and the impacts of biocatalyst purity on product purification

4.1 Introduction

The multi-enzymatic synthesis of X5P was previously described by Zimmermann *et al.*, (1999) where fructose 1,6 bisphosphate was used as a starting substrate to produce the product over a 48-hour period. The time-scale and yield of the process described have so far made it unattractive for industrial use. It was shown in Chapter 3 that a great deal can be learned within the shortest time frame by taking a systematic approach to the characterisation of the reaction. Based on the characterisation different aspects of X5P preparation were described and alternative production methods were put forward where G3P and DHAP were the starting substrates rather than Fru1,6BP. The aims of this chapter are first to put the knowledge gained in Chapter 3 to engineering use and develop lab-scale bioconversion. Particular attention will be given to the requirements of the production phase including the starting substrate, enzyme purity and process conditions. The downstream processing and product recovery will be clearly described here for the first time together with alterations made based on the characterisation results. One of the primary objectives of this study is to identify the key factors that come into play when scaling up a multi-enzymatic process. The scale-up is in aid of increasing the intensity of the X5P process (improving productivity, kinetics and thermodynamics). The production and purification methods shown here will provide the basis for further process engineering and analysis of this model multi-enzymatic process. To achieve the aims of this chapter the following steps are taken:

1. Implement the guidelines and the information gathered on the system to devise a number of lab-scale production processes.
2. Produce the product in the bioreactor at laboratory scale.

3. Conduct a lab-scale purification of the resulting biotransformations to obtain X5P.
4. Analyse the lab-scale processes in terms of their practicalities paying particular attention to the biocatalyst purity and how it impacts the product purity and DSP.
5. Gain information from the biotransformations for subsequent process synthesis, assessment and selection.

The findings in this chapter will enable more informed decisions to be made on by way of process development. At the end of this chapter enough information will be available to rule out further unattractive process options.

4.2 Materials and methods

4.2.1 The purification of transketolase

The characterisation studies showed that the production of X5P was possible very efficiently using pure transketolase and G3P. Pure transketolase was not available in large quantities commercially therefore the crude transketolase derived from *E. coli* JM 107/pQR711 fermentations had to be purified to provide purified transketolase (Tkp). The purification of transketolase at lab-scale was derived from methods described in literature (Littlechild *et al.*, 1995; Hobbs *et al.*, 1996 and Mitra *et al.*, 1998). The purification of transketolase follows the course shown in Figure 4.1.

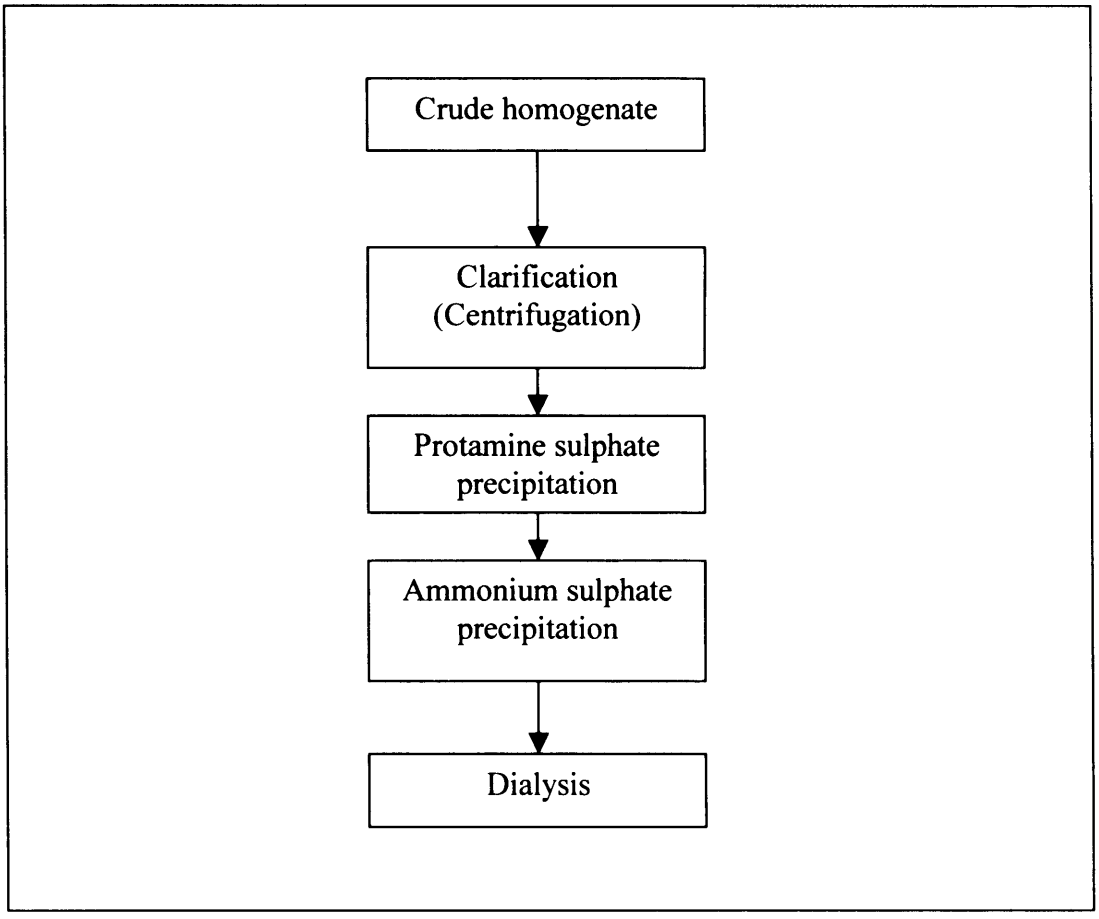


Figure 4.1 The purification of transketolase

It is important to add that after the dialysis stage it is possible to carry out an ion-exchange FPLC, Gel filtration FPLC and crystallisation of transketolase. These purification steps were not carried out in this investigation.

4.2.1.1 Initial purification of transketolase

The transketolase was initially purified (clarified) using a typical protein purification procedure largely based on general methods described in the literature (Hobbs *et al.*, 1996). The cells were produced using the methods described in Chapter 2. The initial stages of purification are also indicated in Section 2.2.6. The transketolase produced at this stage was crude and labelled Tki. In order to obtain pure transketolase, samples were passed through protamine sulphate precipitation, ammonium sulphate precipitation and dialysis.

4.2.1.2 Protamine sulphate precipitation

0.05 % w/v protamine sulphate (Grade X from Salmon, Sigma-Aldrich co., Poole, Dorset, UK), (0.05g in 100mL) was added to the clarified crude extract. The mixture was stirred at 4 °C in a bioreactor with a cooling water jacket for 30 minutes. Thereafter the precipitate was removed by centrifugation at 13000 rpm for 30 min (Biofuge 13, Heraeus Sepatech, Brentwood, Essex, UK) and the supernatant was tested for transketolase activity using the HPLC.

4.2.1.3 Ammonium sulphate precipitation

The supernatant from the centrifuged homogenate was then subjected to bulk precipitation. A wide range of ammonium sulphate concentrations was tried initially (30% - 80%). To develop the precipitation method at each step the pellet (precipitate) was resuspended in buffer, dialysed and tested for transketolase content. 45% (w/v) ammonium sulphate (86 g L^{-1}) was the saturation added to the supernatant solution and this was left standing for 30 minutes at 4 °C in a bioreactor with a cooling jacket. Precipitate was removed by centrifugation at 13000 rpm for 30 min (Biofuge 13, Heraeus Sepatech, Brentwood, Essex, UK) and the clarified supernatant containing Tk transferred to another vessel and made up to 75% (w/v)

saturation. The mixture was stirred at 4 °C for another 30 minutes. The precipitate formed contained transketolase and was removed by centrifugation (13000 rpm, 30 min). The pellets formed by centrifugation contained transketolase and were moved to the subsequent DSP stage of dialysis.

4.2.1.4 Dialysis

The precipitate was resuspended in buffer (10mM Tris-HCl, pH 7.6) and sealed in pre-prepared dialysis tubing (Medicell International Ltd., UK) prepared by boiling in 5% w/v sodium bicarbonate ($NaHCO_3$) and 50 mM ethylene diamine tetra acetic acid (EDTA) for 30 minutes. The prepared tubing was then filled with the resuspended transketolase after ammonium sulphate precipitation. The enzyme dialysis tube finally contained Tris-HCl buffer (10 mL ; 10mM, pH 7.5), ethylenediamine tetracetic acid (EDTA) (1mM), mercaptoethanol (6mM) phenyl methyl sulphonyl fluoride (PMSF) (0.1mM) and benzamidine (0.1 mM). This tube was then left in 2L of low molarity Tris-HCl Buffer (10mM) at 4 °C for two hours. The buffer was changed twice.

4.2.2 Lab-scale preparation of xylulose 5-phosphate

Firstly the transketolase was prepared to the required purity using the methods described in Sections 2.2.6 and 4.2.1. It has been reported in literature that pre-incubating transketolase with cofactors could minimise the lag phase of the reaction (Brocklebank *et al.*, 1999). To confirm the results the transketolase was preincubated with the co-factors for increasing periods of time. For lab-scale biotransformations triosephosphate isomerase (TPI) was added to the reactions requiring the conversion of DHAP to G3P this was the case when purified transketolase (Tkp) was used. Hydroxypyruvic acid (HPA) was added in molar excess according to the amounts investigated in Chapter 3. As shown in the preliminary findings X5P could also be produced starting from G3P and DHAP. Depending on the type of reaction either DHAP or G3P was added as starting substrate. These biotransformations were carried out at lab-scale to confirm the results in Chapter 3 and to magnify any previously unknown issues. The scale of the reaction vessel was 250 mL to compare directly with previous lab-scale research on xylulose 5-phosphate (Zimmermann *et al.*, 1999). The reactions were started

and monitored with the addition of the starting substrate. Temperature was kept at 25 °C and the pH controlled at 7.0 using the pH stat. The reaction vessels were baffled and the stirrer speed kept constant throughout reactions. Table 4.1 illustrates the typical values of components used in the reactions.

Component	Concentration ($g.L^{-1}$)	Concentration (mM)
DHAP	2.55	15.00
G3P	2.55	15.00
HPA	3.19	30.67
TPP	1.10	2.40
$MgCl_2.6H_2O$	1.83	9.00

Table 4.1 Typical reaction amounts of reaction components used in X5P production.

4.2.3 Bioreactor design and reaction conditions

The details of the reactions are fully explained in Section 4.2.2 and their monitoring described in Chapter 2. The attributes of the bioreactor were amended based on the findings in Chapter 3. The mode of the reaction was kept as batch and the substrate concentrations were kept below inhibitory levels in a form to work as fed-batch. The pH was kept at 7.0 using the pH stat apparatus to reduce the degradation of the substrates and product. This is also within the optimal pH range for transketolase (Mitra *et al.*, 1998). The bioreactor was baffled and agitated at a constant rate.

4.2.4 Purification procedure for xylulose 5-phosphate

Zimmermann *et al.*, (1999) have reported the most recent purification method for X5P. The methods used here are an adaptation of those described in the literature.

4.2.4.1 Cation exchange (stage 1)

HPLC analysis confirmed the reactions completion. Reactions were stopped with the addition of a cation exchange resin, 40 g/L (Dowex AG 50W –X8, H^+ form) to the reaction mixture (recommended value by resin manufacturer, Bio-Rad Laboratories Ltd. Hemel Hempstead, Hertfordshire, UK). The resin was gently stirred in the reaction vessel for 1 hour. There was a pH shift observed at the point of adding the cation exchange resin where pH dropped from 7.00 to 1.82. This was the point at which protein in the reaction mixture precipitated and the reaction stopped.

4.2.4.2 Dilution and Filtration (Stage 2)

The reaction mixture was diluted with 250 mL water (equal to reaction volume). This aided the cation exchange and the subsequent filtration of the precipitated protein and resin beads. The cation exchange resin beads were removed from the mixture by a filtration step using porosity 2 glass filter. The protein precipitate was removed using porosity 3 glass filter. For analytical purposes the precipitate was removed using a bench centrifuge at 13000 rpm for 15 min (Biofuge 13, Heraeus Sepatech, Brentwood, Essex, UK). The filtrate was then degassed in preparation for the next stage of anion exchange.

4.2.4.3 Addition of anion exchange resin (Stage 3)

Anion exchange resin AG 1-X8 formate form was added to the solution at 5 grams per 100 mL as recommended by manufacturer (Bio-Rad Laboratories Ltd. Hemel Hempstead, Hertfordshire, UK). The resin was incubated in the solution containing the reaction components by gently stirring at 25 °C for one hour. The solution was at pH 10 for this period therefore it was important not to exceed this period, as the components were rather unstable at this pH.

4.2.4.4 Column separation (Stage 4)

The anion exchange resin containing the reaction components was packed in a glass column (60mL). Methods in literature recommend the use of 1.3 M formic acid for the elution of the reaction components from the anion exchange resin. This was however found to degrade the product rapidly. The product was therefore eluted with Triethyammonium formate buffer (pH 6.95) at the flow rate of 2ml/min. 5mL

fractions were eluted from the column with the more favourable buffer and tested for presence of product and other reaction components using the HPLC method described in Chapter 2.

4.2.4.5 Evaporation and concentration (stage 5)

5mL fractions were tested for product using HPLC and those containing X5P were then combined and evaporated using a Büchi rotavapor R110 (Büchi Labortechnik AG, Flawil, Switzerland). A light yellow mixture resulted containing X5P. This concentrate was 1/3 volume of the combined fractions containing X5P and was at pH 6.95. Figure 4.2 shows the complete purification procedure.

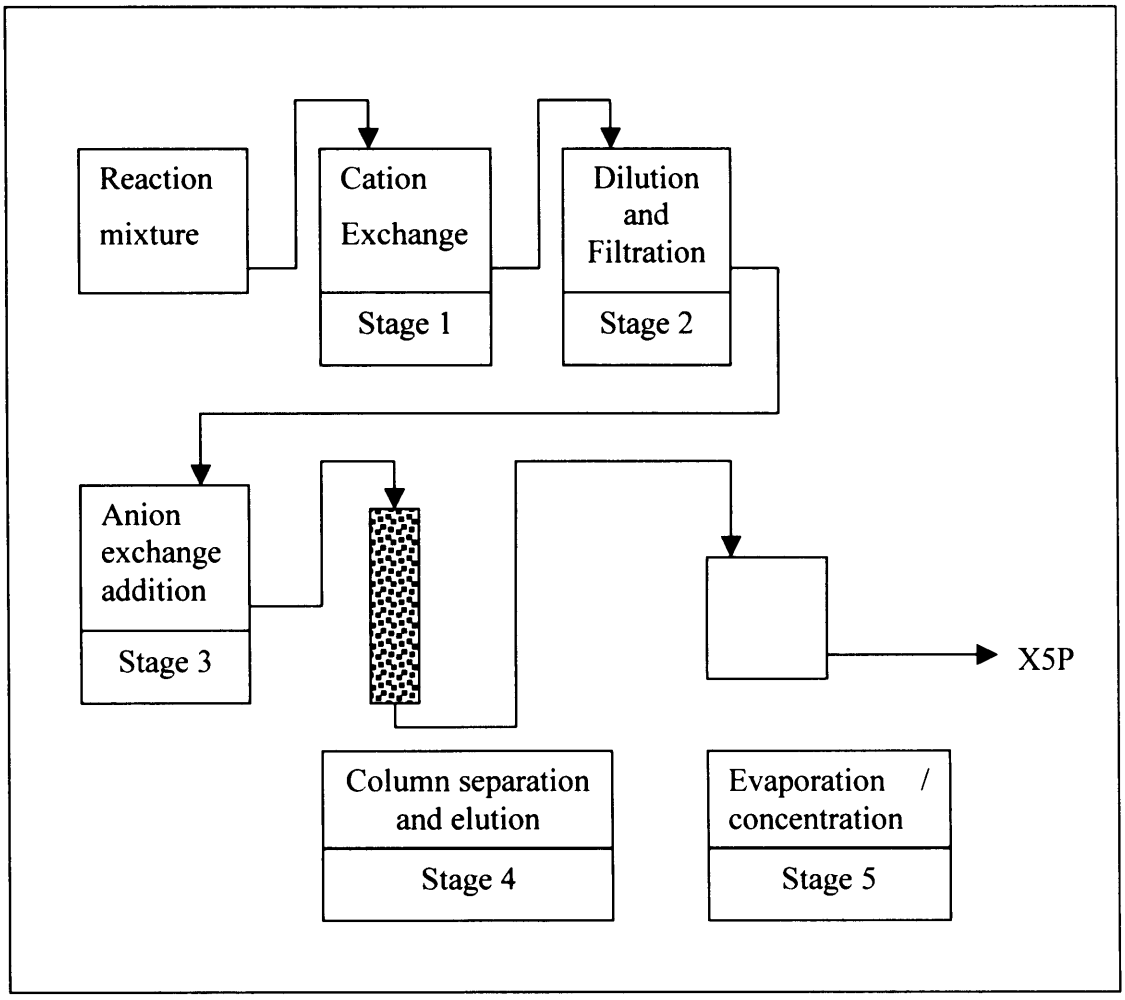


Figure 4.2 The downstream processing stages for purifying X5P.

4.3 Results

4.3.1 Laboratory scale X5P production

The experiments carried out in this part of the investigation were largely based on the findings in Chapter 3. In the previous chapter the results showed that there is a difference between the preparation of X5P when using G3P or DHAP as a starting substrate. The processes performed differently in terms of yield, productivity, kinetics and thermodynamics. DHAP reactions showed better profiles at higher concentrations and G3P reactions showed better performance at low concentrations. As there were no examples to draw from in literature, in this chapter it was important to implement the reactions at lab-scale to verify the results gathered at small scale. This was the first time X5P was being produced from G3P or DHAP rather than Fru1,6BP and it was important to draw a comparison between the two pathways for subsequent process development.

From Figures 3.22 and 3.23 it was clear that the reactions showed similarities in terms of thermodynamics and kinetics at 50 mM concentrations where the processes fell close to each other on both graphs. Due to the limited amount of substrate available particularly with respect to G3P the lab scale processes were run at lower concentrations (15 mM and 20 mM). This meant that there was a gap and a difference between the processes starting with G3P and those starting with DHAP (Figure 3.23) where DHAP reaction needed to be improved or optimised to compare directly with the G3P reaction. Optimisation of the process was possible by compromising the levels of enzyme in the system. This required the use of pure transketolase. It was summarised in the previous chapter that changing the TPI to Tk ratio has a direct influence on the results of the biotransformation. G3P reactions also performed better with pure biocatalyst (Tkp).

Studying the impact of enzyme purity in this chapter therefore became crucial as the biotransformations in this part of the work were to be carried out at larger scale than those previously. It was important to carry out biotransformations with Tkp and Tki starting with G3P and DHAP to understand the tradeoffs between biocatalyst purity and starting substrate. In this chapter transketolase was purified in the laboratory due to the large amounts needed. Therefore the pure transketolase (Tkp) was to be derived from the crude fermentation extracts. These purification experiments were

deemed necessary to identify how each step of purification impacted the transketolase activity.

4.3.2 Transketolase purity

Transketolase was purified as described in Section 4.2.1. After each stage of transketolase purification the activity was tested using the activity assay described in Chapter 2. Transketolase activity increased with each purification step. The following Table 4.2 shows how the crude extract activity was improved by subsequent purification. The purification factor was calculated based on the specific activity of the crude extract. The results showed that the activity of transketolase improved from 0.12 Umg^{-1} by a purification factor of approximately 2 in the first stage of protamine sulphate purification. Ammonium sulphate precipitation did not show a large improvement with a purification factor of approximately 2.5 indicating that the first stage of protamine sulphate precipitation removed most of the impurities. Protamine sulphate precipitation was a rather expensive purification stage and perhaps very costly to implement at large scale.

Purification stage	Activity (μmoles. min^{-1} mg^{-1})	Purification factor
Crude extract (Tki)	0.12	1
Protamine sulphate precipitation	0.27	2.25
Ammonium sulphate precipitation	0.32	2.66
Dialysis	0.43	3.58

Table 4.2 The increase of transketolase specific activity (μmoles. min^{-1} mg^{-1}) based on substrate glycolaldehyde as a result of each purification stage indicating an increasing purification factor.

The purification factor represents the specific activity after each stage of enzyme purification divided by the specific activity in the crude extract (Tki) and after dialysis this value was above 3.0 and the specific activity of the Tk close to 0.5 U

mg^{-1}. This value was similar to those obtained with commercially available Tk consequently no further purification was carried out in this study. Adding or removing purification stages was possible at this stage and further study is needed to fully describe the purification of transketolase. It was at this point however more important to carry out the biotransformations and obtain Tkp in the shortest possible stages.

4.3.3 Reducing the lag phase

Previous studies have suggested that by pre-incubating transketolase the lag phase of the reaction can be drastically reduced (Brocklebank *et al.*, 1999). In this experiment erythrulose production was used as a model to confirm this. Figure 4.3 shows the results of the experiment. It was described by the results that the yield of product suffered due to a lag phase if transketolase is not pre-incubated with the co-factors for a minimum of 20 minutes. By pre-incubating the enzyme with the co-factors the specific activity is improved greatly. The specific activity is at the highest level after 40 minutes incubation however there is not a great difference between incubating for 20 minutes or 40 minutes. Beyond 20 minutes (40 and 60 minutes) no major benefits in terms of specific activity were observed. At 60 minutes there was a drop in activity, which may be due to the increased instability of holo-transketolase. Previous studies suggest a preincubation period of 1hr 30 min (Hobbs *et al.*, 1996) however the results here indicated that the enzyme is fully functional after 20 minutes. This pre-incubation period is a crucial preparative step for the transketolase reaction. It is also important to study the impact of increasing cofactor concentration to fully describe the pre-incubation of Tk. Cofactor concentration experiments were not carried out in this study.

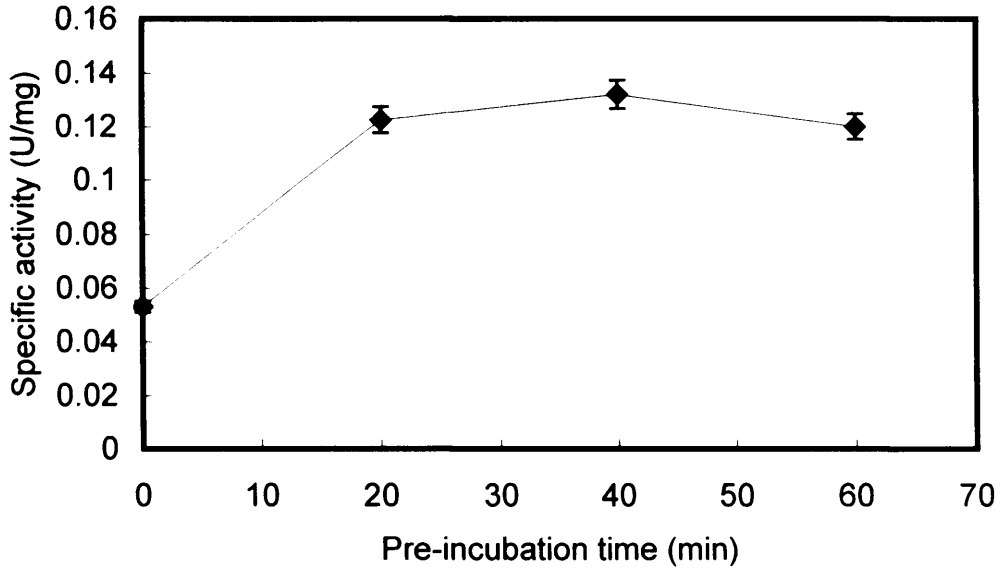


Figure 4.3 The importance of the pre-incubation of transketolase with cofactors TPP and Mg^{++} indicating an increased initial activity (—◆—) after 20 minutes considering the control without cofactors (apo-form).

4.3.4 Starting substrate

Depending on the starting substrate the kinetics and the thermodynamics of the reaction changes (Chapter 3). Running reactions starting with DHAP will result in different yields, kinetics, thermodynamics and productivities than running reactions starting with G3P. Based on the previous characterisation studies experiments were conducted with purified (Tkp) and crude transketolase (Tki) extracts. These forms of enzyme preparations were applied to each starting substrate (G3P and DHAP). In the case of DHAP TPI needed to be added when using Tkp as a biocatalyst. As stated in Section 4.3.1 the lab-scale biotransformations were run at low concentrations. Although this meant that the processes would experience different profiles it eliminated any possibility of product or substrate inhibition and meant that the two starting substrates could be compared. The flowsheets properties are shown in table 4.3. The resulting yields, kinetics, thermodynamics and productivities are shown in table 4.4. A comparison was made with the results

gathered in Chapter 3 especially by adding the results gained from the Fru1,6BP lab-scale results to Table 4.4.

The results gained from the experiments show that the G3P and DHAP reactions can be performed at lab scale and the yields and productivities are comparable to those described in literature and biotransformations developed by the pharmaceutical industry (Straathof *et al.*, 2002). The highest product concentration achieved was 3.2 g L^{-1} starting from 15 mM G3P and with Tkp giving a 94 % yield on substrate. These values are comparable to LY300164 production using a dehydrogenase in industry (Straathof *et al.*, 2002). The biotransformations conducted at lab scale can be described by simple flowsheets (Table 4.3).

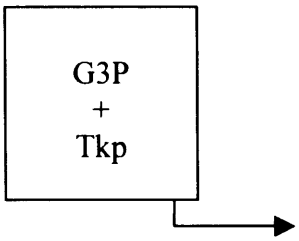
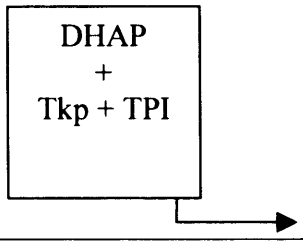
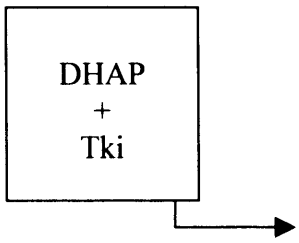
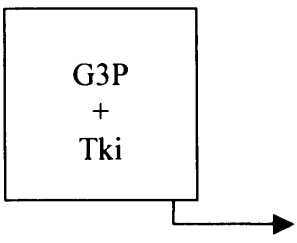
Process flowsheet	Description
	G3P was added to Tkp and HPA pre-incubated with co-factors TPP and Mg^{++}. The product concentration was 3.2 gL^{-1}. The product stream was relatively pure.
	DHAP was added to a mixture of Tkp (1 UmL^{-1}) and TPI (20 UmL^{-1}). The resulting product concentration was 2.5 gL^{-1}. The product stream was relatively pure with some remaining DHAP.
	DHAP was added to crude transketolase (Tki) with activity of 1 UmL^{-1}. The resulting product concentration was 1.8 gL^{-1}. The product stream contained by-products and remaining substrate.
	G3P was added to crude transketolase (Tki) and the resulting product stream contained 2.43 gL^{-1} X5P.

Table 4.3 Lab-scale biotransformations flowsheets and properties

By comparing the results of the results at lab-scale to those obtained in Chapter 3 it was possible to understand the impact of scale-up on the processes. The yields, productivities, kinetics and thermodynamics suffered at larger volumes (Table 4.4). This was probably due to mass transfer issues in larger bioreactors. When comparing the lab-scale reaction conducted at the start of the investigation starting with Frul,6BP it is clear that the productivity or STY (0.07 g/L/hr) is very much lower than the G3P (STY of 1.79 g/L/hr) and DHAP (STY of 0.37 g/L/hr) processes. The results confirm that the process was rightly eliminated in the characterisation stages.

Reaction	X5P (mM)	Substrate (mM)	Yield [P]/[S]	Yield [P]/[E]	STY (g/L/hr)	Initial rate (g/L/hr)
G3P → X5P + Tkp	14.04	15	0.94	1.61	1.79	31.67
DHAP → X5P + Tkp + TPI	11.04	15	0.74	1.27	0.67	4.18
DHAP → X5P + Tki	8.05	15	0.54	0.93	0.37	1.87
G3P → X5P +Tki	10.56	15	0.70	1.22	0.92	4.79
DHAP → X5P + Tki	13.40	20	0.67	1.54	0.76	3.76
G3P → X5P +Tki	15.20	20	0.76	1.75	0.86	4.24
Fru 1,6 BP → X5P +Tkp + TPI	15.24	20	0.76	1.76	0.07	0.10

Table 4.4 Comparing the lab-scale resulting yields, productivities, kinetics and thermodynamics with regards to transketolase purity and starting substrate.

By analysing the results from the lab-scale biotransformations soon a problem emerged. Processes incorporating Tki as a biocatalyst suffered in terms of product concentration, kinetics, productivity and thermodynamics. The results for lab-scale reaction ran using DHAP with Tki are shown in Table 4.4 and Figure 4.4. This is the first time that X5P has been produced in this manner at this scale and previously in literature crude transketolase has been used in biotransformation studies without any mention of problems in terms of yield and productivity (Chauhan *et al.*, 1997; Hobbs *et al.*, 1996; Brocklebank *et al.*, 1998; Mitra and Woodley, 1998). Starting with G3P as a primary substrate and Tkp the resulting product solution showed approximately 15mM concentration of X5P (3.2 g/L). In the case of DHAP and Tki the product concentration was 8.05 mM (1.8 g/L). The yields are lower than that for reactions run with G3P. This difference in the DHAP and G3P processes was predicted in Chapter 3 and was partly attributed to the ratio of TPI to Tk found in crude transketolase. Previous reaction results indicated that the DHAP process may be improved by adding Tkp to the reaction. However the results gathered using semi-purified transketolase highlighted a more important issue. The presence of other enzymes in the crude mixture means that the components are vulnerable to side reactions. Consequently the process may be rendered inefficient. The product might be broken down or the substrates used to produce unwanted products. This was proved to be the case in the crude Tk reactions (Figure 4.5).

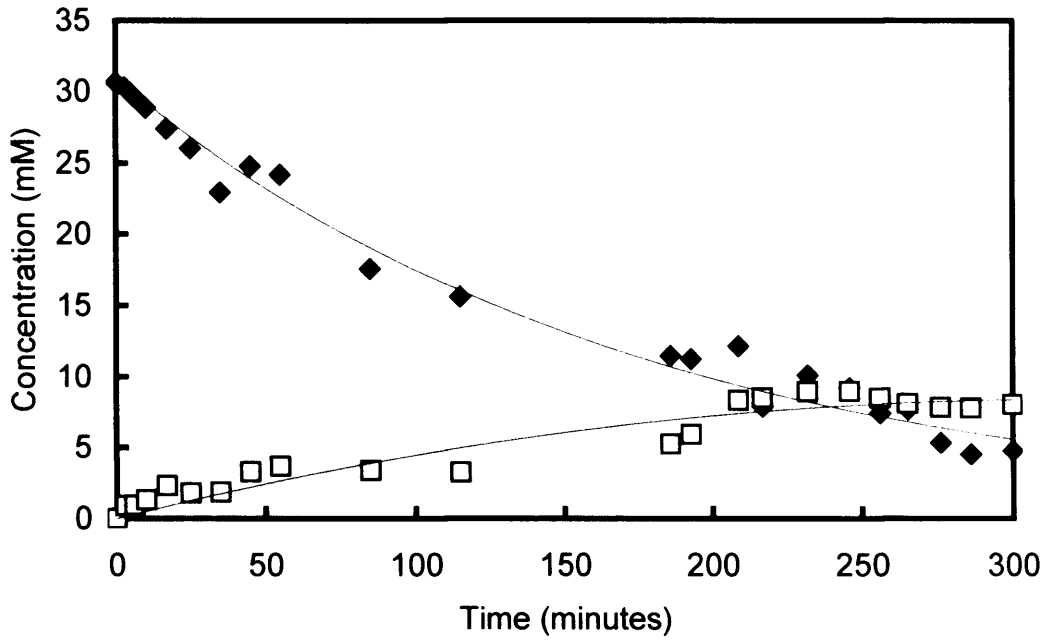


Figure 4.4 The production of X5P from 15 mM DHAP using Tki, indicating HPA depletion (-◆-) and X5P accumulation (-□-) in mM.

4.3.5 Side reactions due to the use of semi-purified enzyme

The scaling up of the processes and the use of electrochemical HPLC detection showed more detail about the model multi-enzymatic process. The presence of by-products/contaminants can be observed in the form of peaks in the HPLC chromatographs. The unidentified peaks were recorded and plotted in Figure 4.5. The emergence of these peaks is not observed in experiments involving purified transketolase (Tkp) as a biocatalyst. From Figure 4.5 it is clearly distinguishable that as the X5P production comes to an end the by-product production reaches maximum rate at 200 minutes into the reaction. These components are named by-products, contaminants or impurities as a result of unwanted side reactions. Further detailed analysis of the samples was carried out to display the true nature of these anomalies.

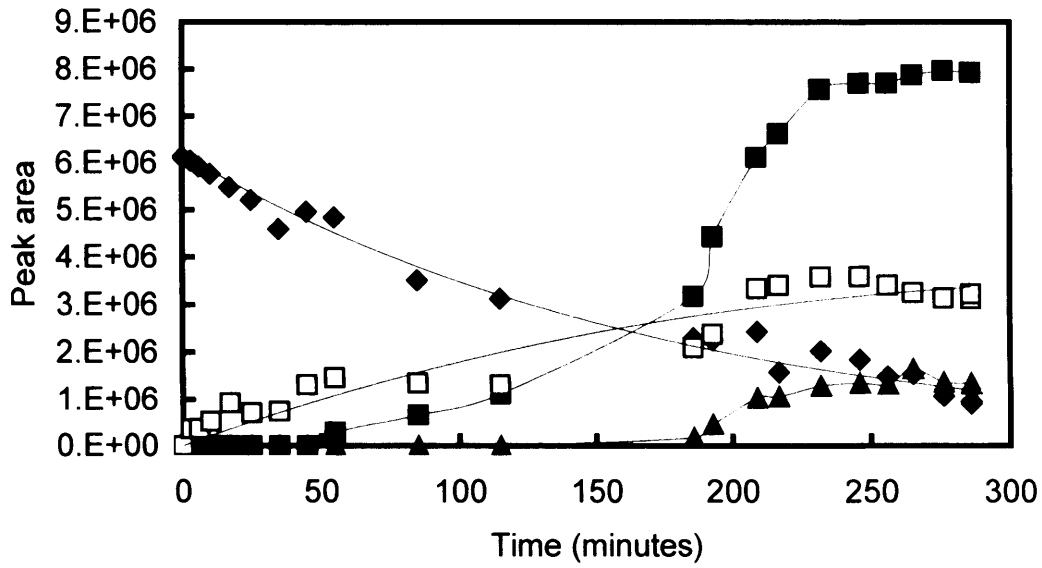


Figure 4.5 The levels of by-product R5P (-■-) and by-product 2 (-▲-) during the production of X5P (-□-) from HPA (-◆-) based on peak area.

The appearance of unwanted side reactions is common in whole cell systems (Ivanetich *et al.*, 1995). It is inevitably an issue when working with impure enzymes. Having analysed the reactions at lab-scale the product containing batches were put through a purification process. This enabled a detailed analysis of the samples at different stages of purification and consequently allowed the identification of the most prominent contaminant (Appendix VIII). As the by-products were not all identified they are represented as peak areas from the HPLC and not actual concentrations. To this end the Peak areas can only be used as an indication of by-product formation and not in terms of concentration. A larger peak area does not necessarily relate to a higher concentration. Peak area is dependent on the type of the compound and a calibration curve is necessary for each individual compound analysed. Therefore in Figure 4.5 it could not be concluded that there was more by-product (R5P) than X5P in terms of concentration.

4.3.6 Purification of product

The method X5P purification is fully detailed in Section 4.2.4 and clearly shown by Figure 4.2. Samples were taken at each stage of the purification process and analysed by HPLC. Figure 4.7 shows the level of all components detectable by HPLC at each stage of the X5P purification in the reaction starting with DHAP as a substrate. It is clear that after the reaction has reached completion there is some remaining HPA and DHAP as well as by-products. As explained earlier this process included the use of impure transketolase (Tki). The results state clearly that by using Tki there is residual DHAP and HPA left in the reactor after the reactions end and that there is also the presence of by-products, which is probably as a result of a side reaction by intrinsic *E. coli* enzymes. Analysing the samples at each purification step showed for the first time what was being removed by each stage of purification (Figure 4.6). The levels of the components at each stage of purification are shown in Figure 4.7 in terms of peak area. At the stage 4 of purification (Anion exchange and elution) samples were taken at intervals and analysed. It was possible to detect very quickly using HPLC which fractions contained the product X5P. These fractions were combined and put through the final stage of purification (evaporation). After the final purification stage a light yellow mixture resulted which contained X5P. In processes involving crude transketolase the presence of the major contaminant were also detected alongside X5P.

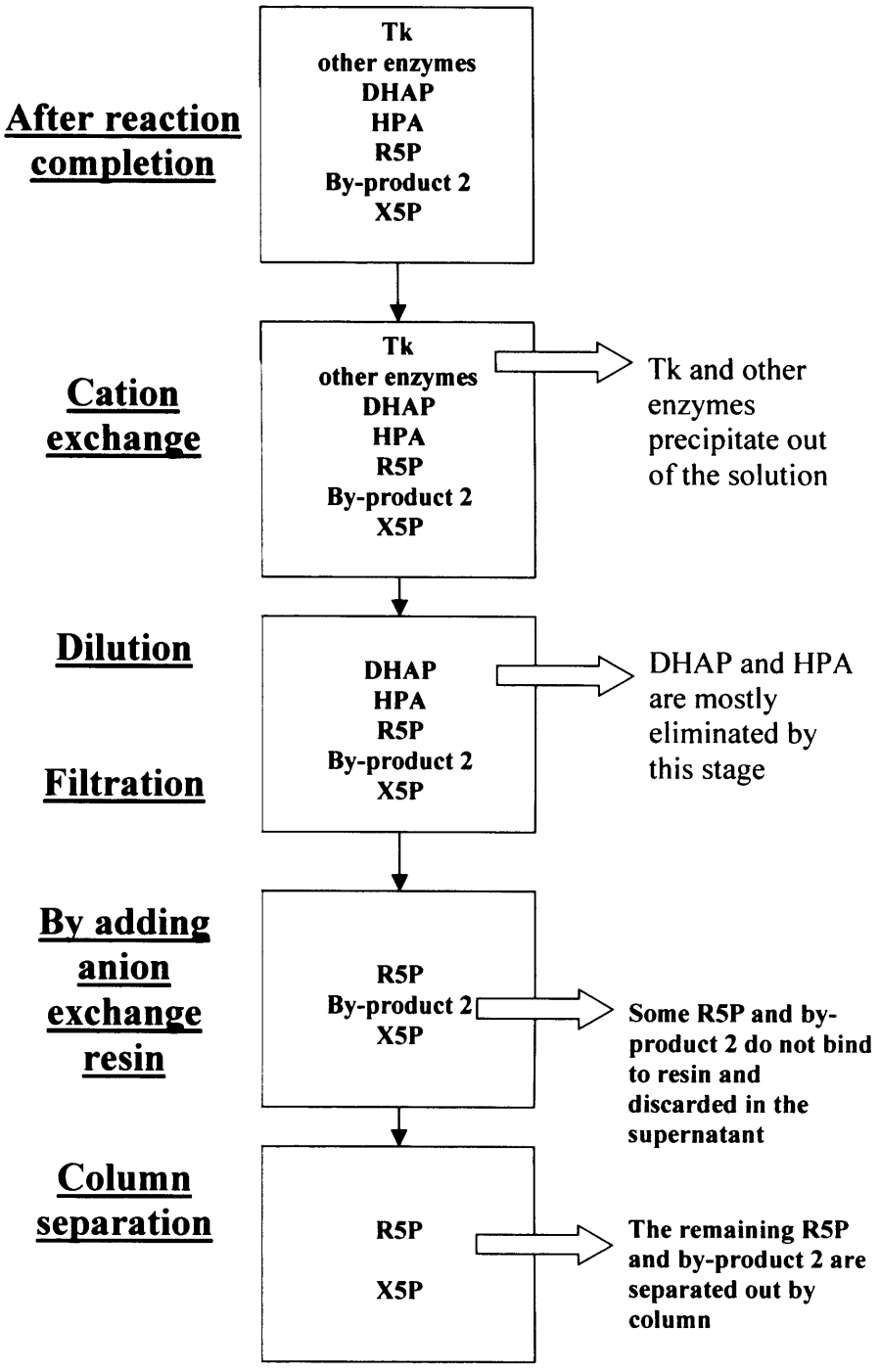
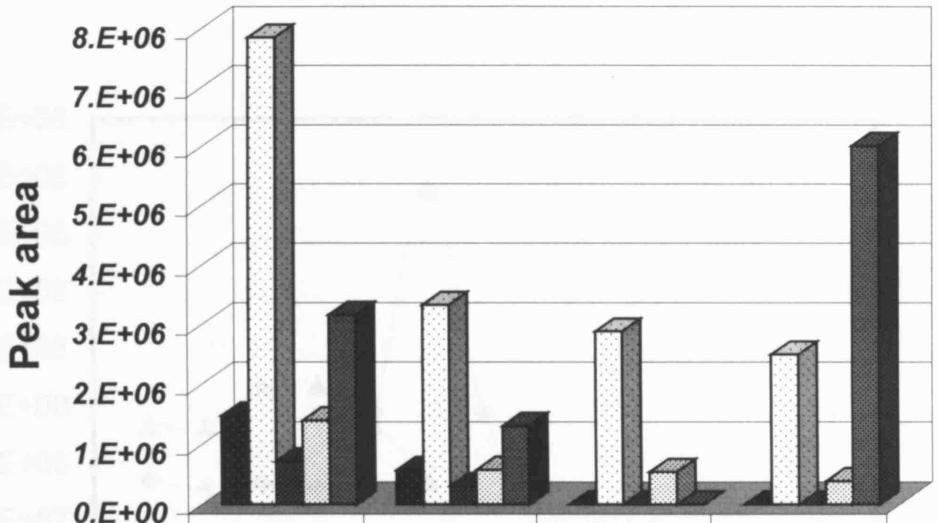


Figure 4.6 The purification stages of X5P and the components removed at each stage



	After Cation Exchange	Dilution and Filtration	Anion Exchange Resin Added	Column Separation
DHAP	1487734.5	575050.5	0	0
R5P	7859010	3342281	2891694	2504038
HPA	723034.5	306425	0	0
By-product 2	1404033.5	580146	546090.2	388893
X5P	3175064.5	1313788.5	0	6027650

Figure 4.7 The levels of DHAP, HPA, X5P, R5P and by-product 2 in each stage of the X5P purification procedure.

It is clear from Figure 4.7 that the levels of X5P increase by the purification process. There is some residual R5P in the end product stream. This is due to the impure source of biocatalyst used. The analysis of the fractions from the elution stage of the purification is shown in Figure 4.8. It is visible from this plot that the by-product R5P eluted off first. X5P is eluted off the column after the contaminants and thus it is possible to separate the two. This laborious and expensive purification stage is necessary only to ensure X5P purity when by-products are present (when Tki is used). This stage would not be required if the product stream was pure as with using purified transketolase. The number of X5P downstream stages reduces as the source of enzyme becomes purer. Samples were taken from the elution stage, freeze-dried and analysed by NMR to identify the nature of the contaminants.

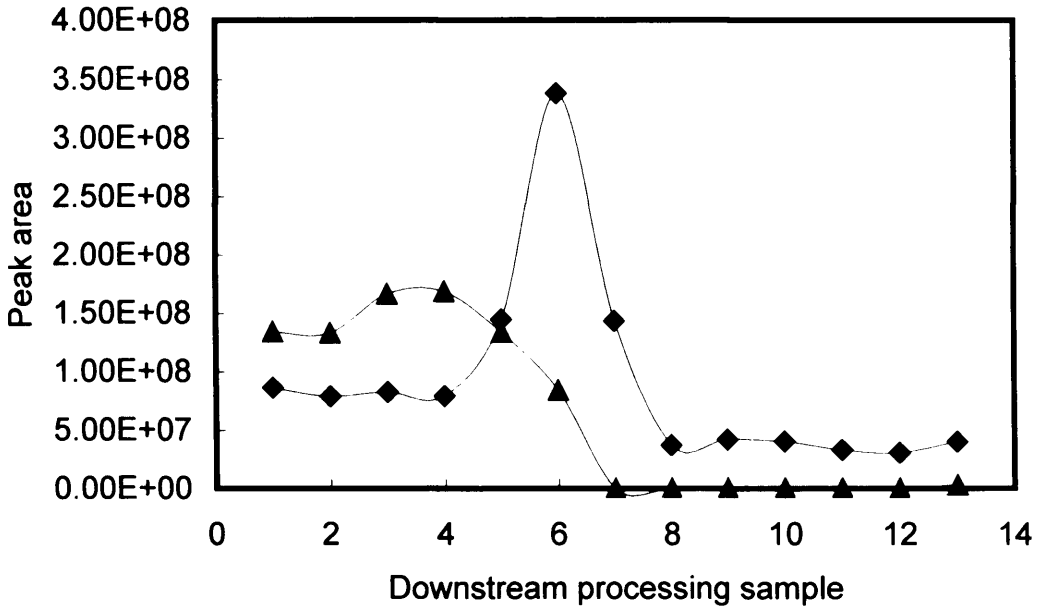


Figure 4.8 The elution of reaction components from the anion exchange column, indicating levels of X5P (—◆—) and R5P (—▲—).

4.3.7 Identification of lab-scale constraints

Based on the lab-scale experiments the enzyme purity became an important issue to consider. Those reactions containing impure Tk (Tki) showed levels of by-product formation. Taking samples from the anion exchange stage of purification, drying them and performing NMR analysis enabled the identification of the by-products. It was not possible to analyse by-product 2 as it degraded very rapidly prior to NMR analysis.

The Ms data from using +ve and -ve ES show different peaks, one at 122, and the other at 96 and 112.Mass of non-phosphorylated X5P is 150. Thus the -CO peak at 122, suggests the peak is or an isomer of X5P, ribulose 5-phosphate. This fragmentation was similar to one observed by Zimmermann *et al.*, 1999. It was concluded at this point that the major contaminant in the reaction was ribulose 5-phosphate (R5P). (See Appendix VIII for NMR analysis). Together with NMR

analysis it was clear that there was a constraints on the reactions in terms of enzyme purity.

4.4 Discussion

The primary aim of this work was to gather and apply all the knowledge gained on the model multi-enzymatic system in Chapter 3. The practicalities of running the X5P process at lab-scale were considered as well as the impact of biocatalyst purity on product DSP. Laboratory scale biotransformations were carried out with particular attention given to the guidelines provided by the characterisation results. For the first time an analysis with regards to purifying X5P was attempted. With scaling up the processes to laboratory level certain issues came to the foreground that were previously not considered in the characterisation stages. Scaling up this model system magnified issues such as biocatalyst purity and starting substrate. The findings are here discussed with greater attention to multi-enzymatic processes as a whole and the impact of the findings on process development.

4.4.1 The purification of transketolase

In the purification of transketolase at the stage of dialysis the protease inhibitor phenyl methyl sulphonyle fluoride (PMSF) was used which inhibits both trypsin and chymotrypsin (serine proteases). Benzamidine was also added to inhibit trypsin-like serine proteinases. The half life of PMSF and benzamidine is an important consideration however the two hour period for dialysis has been reported too short for the residual salt to leave the transketolase sample. Reports suggest a 12-hour dialysis period (Hobbs *et al.*, 1996).

4.4.2 The lab-scale production of X5P

Increasing the intensity of the biotransformations was necessary to confirm the findings in the characterisation stage. This scaling up also indicates the relevance of the information gathered in the characterisation stage to eventual process development. Process efficiency is often addressed in terms of yield, product concentration and productivity (Straathof *et al.*, 2002). Subsequently a comparative quantitative analysis of the technical parameters of the X5P process at lab-scale was made (Tables 4.3 and 4.4). The results were used to identify whether the proposed

system characterised in Chapter 3 has the potential for development into a competitive process. Based on the guidelines in Chapter 3 the processes should be limited in substrate concentration. This was to keep substrate inhibition minimised. This was considered acceptable as long as the productivities were kept close to the reported volumetric productivities of biotransformation processes (Straathof *et al.*, 2002). It has also been noted in literature that the starting aldehyde, causes deactivation of the enzyme' thereby placing a limit on the concentration of the starting material that can be used (Turner, 2000). The purity of transketolase was not fully addressed in the characterisation stage. From the findings in this, G3P reactions benefit from the use of purified transketolase (Tkp). This justified the purification of transketolase for the reaction. The effects of using pure transketolase were studied by running lab-scale biotransformations. The results showed that using pure transketolase to change the TPI/Tk ratio might give way to better DHAP conversions.

More importantly the repercussions of using impure transketolase were highlighted by the case study shown in Section 4.3.5. It was important to eliminate any possibility of a lag phase. The pre-incubation experiment proved that sufficient time must be allowed for transketolase in the presence of the cofactors. Having conducted the biotransformations at large scale it was possible to observe the plots and compare the kinetic data obtained from the reactions. The results showed that when Tki is used the production of unwanted by-products occurs midway through the reaction. This was an important finding and for the first time explained the poor mass balances. Some of the substrate/product was being converted to unwanted by-products. It was not possible to identify the exact nature of the by-products using HPLC techniques. Purification of the production stream was necessary to identify and separate out the by-product as well as obtain X5P in pure form. The reactions involving Tki were less laborious practically as the processes did not need enzyme purification and the time to product was drastically reduced. However as the results clearly convey this had a negative impact on the overall success of the reaction. The processes containing purified enzyme were much cleaner ones. This was visible in the bioreactor and confirmed by HPLC results. However the cost and the time and energy requirement for purifying the biocatalyst was a major concern.

4.4.3 The lab-scale purification of X5P

The purification of the final product was deemed a necessary procedure to separate out the unused substrates, the contaminants and by-products. The purification processes involved were for the first time analysed in terms of component levels. It was shown that in the case of using impure Tk (Tki):

- The product stream contains a mixture of product, excess intracellular protein, residual substrates and by-products/contaminants.

- The first stage of purification (cation exchange) removes most of the cell constitutes.

- The second stage (dilution and filtration) removes the protein from the solution (including Tk) by filtration leaving the product and other components in the mixture.

- The third stage (anion exchange) and stage 4 (column separation) of the purification separates out the product from the by-product and contaminants.

- Stage 5 of product purification concentrated the product.

It was shown by the results that the final stages of X5P purification were only necessary in the presence of by-products such as R5P. The presence of such by-products was only detected in biotransformations with Tki. It was highly likely that the crude transketolase contained some biocatalysts found naturally in *E. coli* such as ribulose 5-phosphate 3-epimerase which in turn resulted in the production of R5P (Racker, 1962). This meant that some of the substrate was first converted to X5P and subsequently epimerisation took place rendering the reaction inefficient.

4.4.4 Production phase and downstream tradeoffs

The results and the data gathered here highlighted that the in the scale-up of a multi-enzymatic synthesis a number of priorities must be first addressed. Firstly it is

important to decide on the level of purity of the biocatalysts. An impure enzyme source leads to an impure product stream and therefore adds to downstream costs. Figure 4.9 illustrates the main process options in the lab-scale multi-enzymatic synthesis. First route (route 1, Figure 4.9) is to use a cheap substrate such as DHAP together with a crude extract (Tki). In the second route (route 2, Figure 4.9) the enzyme can be improved upon to reduce downstream costs. In the third route (route 3, Figure 4.9) the purest form of biocatalyst is used together with the substrates leading to the minimum number of product purification stages.

The issue of biocatalyst purity is probably magnified in multi-enzymatic systems. Due to the delicate balance of all the components in the reaction the presence of other biocatalysts can greatly impact a process. As shown in this model system the production of X5P was directly effected by the presence of an unwanted biocatalyst.

It is possible to tackle this problem by:

1. Using the purest forms of biocatalysts and substrates available (increases costs of the process).
2. Gathering more information about the system (increases complexity of the process).

In the second method information must be gathered about all the components in the process. Such as what are the biocatalysts present in Tki exactly and to what activities and levels. Using this information it is possible to balance the process by changing enzyme and substrate levels. For example add more Tk to the system to counteract the problem.

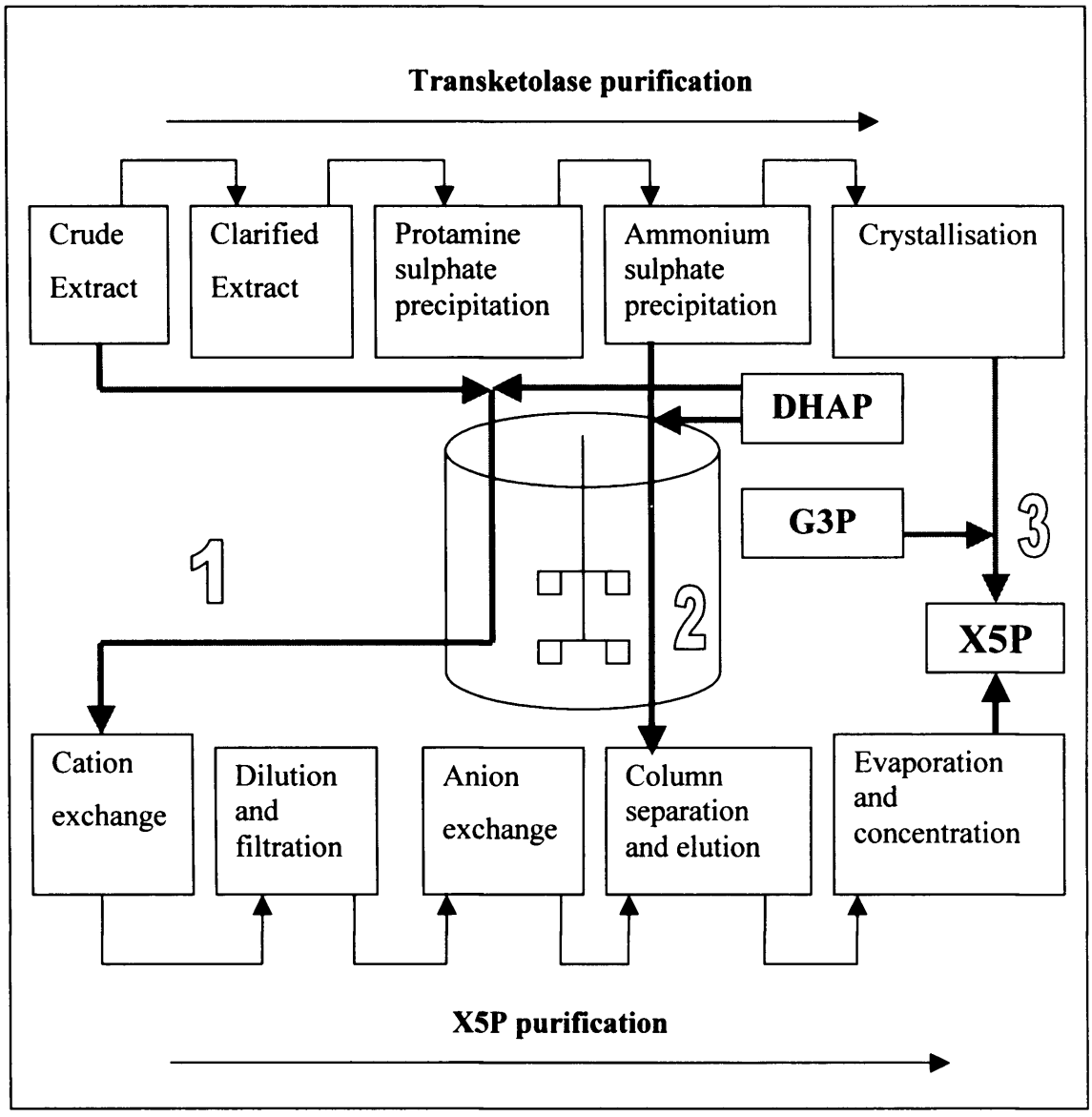


Figure 4.9 The process options for multi-enzymatic preparation of X5P.

The results gathered here convey that there is a true trade-off between using pure biocatalyst and impure biocatalyst. This also depends on the starting substrate. Using G3P for example means that in the presence of pure substrates the product stream does not contain by-products and unused substrate. The kinetics of G3P biotransformations conveyed that the best conversions are achieved using G3P.

This means that less downstream stages are needed to purify the product X5P. DHAP reactions on the other hand are more susceptible to lower conversions and unconverted substrate remains in the product stream. A matrix was constructed to describe this rather complex trade-off between biocatalyst purification and product purification (Table 4.5).

Transketolase purification →

	Crude extract	**Clarified extract**	**Protamine sulphate precipitation**	**Ammonium sulphate precipitation**	**Crystallisation**
Cation exchange	● ○	● ○	● ○	● ○	● ○
Dilution and	● ○	●			
filtration	● ○	●			
Anion exchange	● ○	● ○	●		
Column separation and elution	● ○	● ○	● ○		
Evaporation and concentration	● ○	● ○	● ○	● ○	● ○

Table 4.5 The transketolase purification and downstream processing trade-off where processes using DHAP (●) or G3P (○) as a starting substrate.

Analysing the tradeoffs between preparation and purification it is possible to decide the best rout for the process depending on what substrate is preferred. The choice of substrate is often determined by the economics. It is possible to categorise the substrates here as low value (DHAP) and high value (G3P). G3P was chosen as to represent the valuable substrate as it suffers form instability as shown by studies in the characterisation stage. DHAP was considered the cheaper option as it is now available in large quantities commercially. Figure 4.10 illustrates how making decisions about the substrate; enzyme purification and product purification can affect the final process and what must be considered in greater detail.

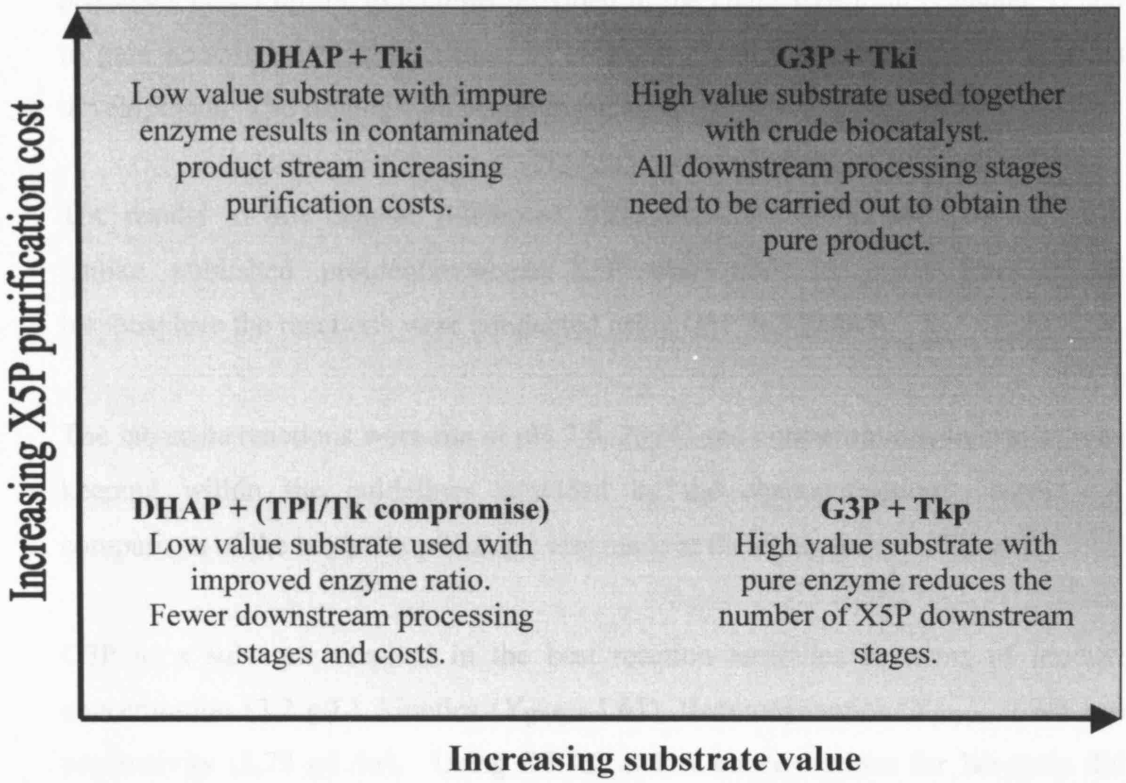


Figure 4.10 The tradeoffs for the X5P processes based on substrate value, the purity of biocatalyst used and how they impact X5P purification costs.

It is clear from the results gathered in this chapter that decisions must be made on what biocatalyst source to use and the starting substrate. These decisions however must take into consideration the tradeoffs between biocatalyst purity (biocatalyst cost), substrate value and the number of X5P DSP stages (product purification costs). Making the right decisions early is essential for rapid process development (Lye *et al.*, 2002). The information gathered in this chapter must be used in a logical manner to develop a set of possible processes for X5P production. From Figure 4.10 it is possible to eliminate the use of impure transketolase and G3P as a process option. As G3P is assumed a valuable substrate the use of Tki cannot be justified in a process.

4.5 Summary

As stated in Section 4.1, it was aimed in this chapter to conduct the model multi-enzymatic system at laboratory scale. This was to determine the performance of the processes based on the guidelines provided in the characterisation (Chapter 3) and to gain knowledge on purification of biocatalyst and product for further process development. The findings are summarised here:

The results in this chapter illustrated alternative lab-scale productions of X5P. Unlike published protocols where X5P was produced using fructose 1,6 bisphosphate the reactions were conducted using G3P and DHAP.

The lab-scale reactions were run at pH 7.0, 25 °C and concentrations below 20 mM keeping within the guidelines provided by the characterisations stages. A comparison of the lab-scale processes was made at these reaction conditions.

G3P as a substrate resulted in the best reaction attributes in terms of product concentration (3.2 g/L), kinetics ($Y_{[P]/[E]}$ 1.61), thermodynamics ($Y_{[P]/[S]}$ 0.94) and productivity (1.79 g/L/hr). Using DHAP al low concentrations for lab-scale did achieved less in terms of product concentration (2.5 g/L), kinetics ($Y_{[P]/[E]}$ 1.27), thermodynamics ($Y_{[P]/[S]}$ 0.73) and productivity (0.67 g/L/hr).

The purification of transketolase was found to be a key factor in the process. When using impure transketolase (Tki) the DHAP reaction product concentration was 43 % below that of the G3P reaction.

The purification of the product stream and subsequent NMR analysis revealed the presence of ribulose 5-phosphate due to running reactions with impure transketolase.

The tradeoffs between biocatalyst purity, starting substrate and product purification were considered. From this set of experiments a further process option was eliminated in the form of using G3P with Tki.

5 Process synthesis and assessment for the production of xylulose 5-phosphate

5.1 Introduction

The results gathered so far in this thesis lay the foundations for a detailed study into identifying and putting forward processes for industrial use. A method of characterising the system in the shortest time was put forward in Chapter 3. The characterisation provided distinct guidelines on conducting the process. The multi-enzymatic system was tested at lab-scale (Chapter 4). The study highlighted the importance of enzyme purity and starting substrate together with guidelines on the levels of substrate and biocatalyst to use. Tradeoffs between biocatalyst purification and X5P downstream processing were addressed. However the information gathered so far is not enough to ensure industrial success of X5P production and the process development is not complete. It is important to use the data and information gathered so far to give specific process options for industry. The primary aim of this chapter is to gather the information learned on the system and design a set of possible processes. Having identified a set of processes the aim is to choose the most viable ones and more importantly to eliminate unattractive options early. Ensuring the success of this type of reaction in industry is difficult without following logical process development techniques. The chemical industry has benefited from a framework of conceptual design and process analysis (Rudd *et al.*, 1973).

It is therefore also the aim of the work presented in this chapter to suggest methods and rules of thumb such as those used in chemical process development. The methods presented here are aimed to provide a framework on how to design a series of possible processes and how to choose the best candidates for the industry. This chapter is concerned with providing a methodology firstly by way of process synthesis where possible reaction paths, flowsheets or processes for the production of X5P are drawn. Secondly a process assessment and selection procedure is suggested. Taking into consideration the process options eliminated in Chapter 3

and 4 the processes designed are assessed towards identifying the best possible industrial options.

5.2 Process synthesis

Based on the theories described in Section 1.4 it is possible to outline the activities required in process synthesis and assessment for process development (Figure 5.1). Process design/synthesis is a complex activity where all the factors involved in a particular reaction have to be taken into consideration. This is an innovative stage where based on the information and data gathered on the reaction process flowsheets are devised. Often a hierarchical approach can be taken (Douglas, 1988). Process synthesis is a sequence of activities towards identifying a valid set of process possibilities, which may be outlined in the form of flowsheets or simple diagrams. After synthesizing a set of processes it is important to assess them. This assessment is based on some basic knowledge about the constraints of the process. This basic knowledge is gained by characterization and preliminary experiments (Chapters 3 and 4). By considering the process attributes the most unattractive options are eliminated and the best ones put forward (process assessment and selection).

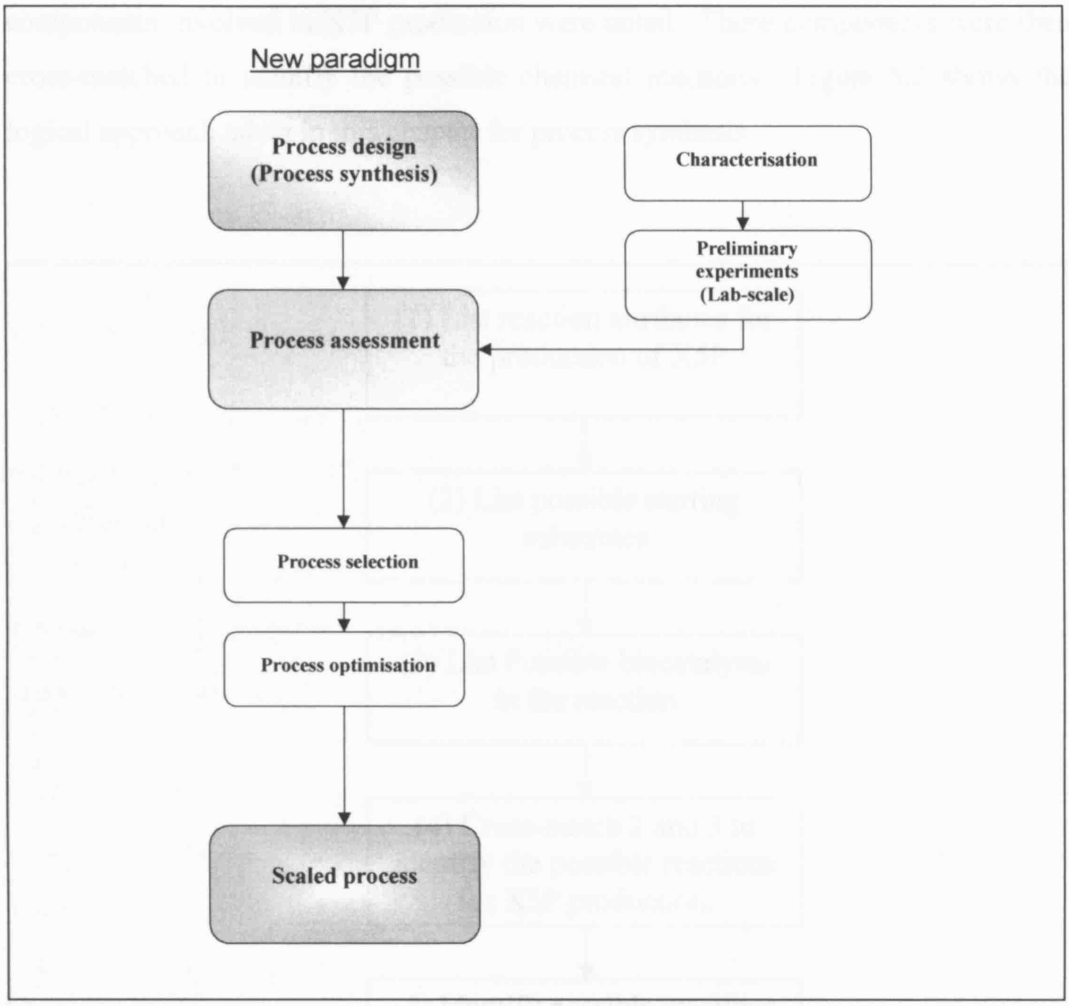


Figure 5.1 Detailed process development by process synthesis and assessment.

For process synthesis some basic information is needed, firstly on the reaction components. There are three possible starting substrates G3P and DHAP and Fru 1,6BP. Process options starting with Fru1,6BP were eliminated in the characterization stage (Chapter 3). The main biocatalysts involved are transketolase, available as a crude extract (Tki) or purified (Tkp) forms. Triosephosphate isomerase (TPI) is also added in pure form to transform DHAP into G3P. TPI does not need to be added in the presence of Tki due to the natural levels present in the *E. coli* cell. The physical properties of all the components and the ideal reaction conditions are known from previous experiments. There are several different possible synthetic strategies. In this primary stage the reaction

components involved in X5P production were noted. These components were then cross-matched to identify the possible chemical reactions. Figure 5.2 shows the logical approach taken in this chapter for process synthesis.

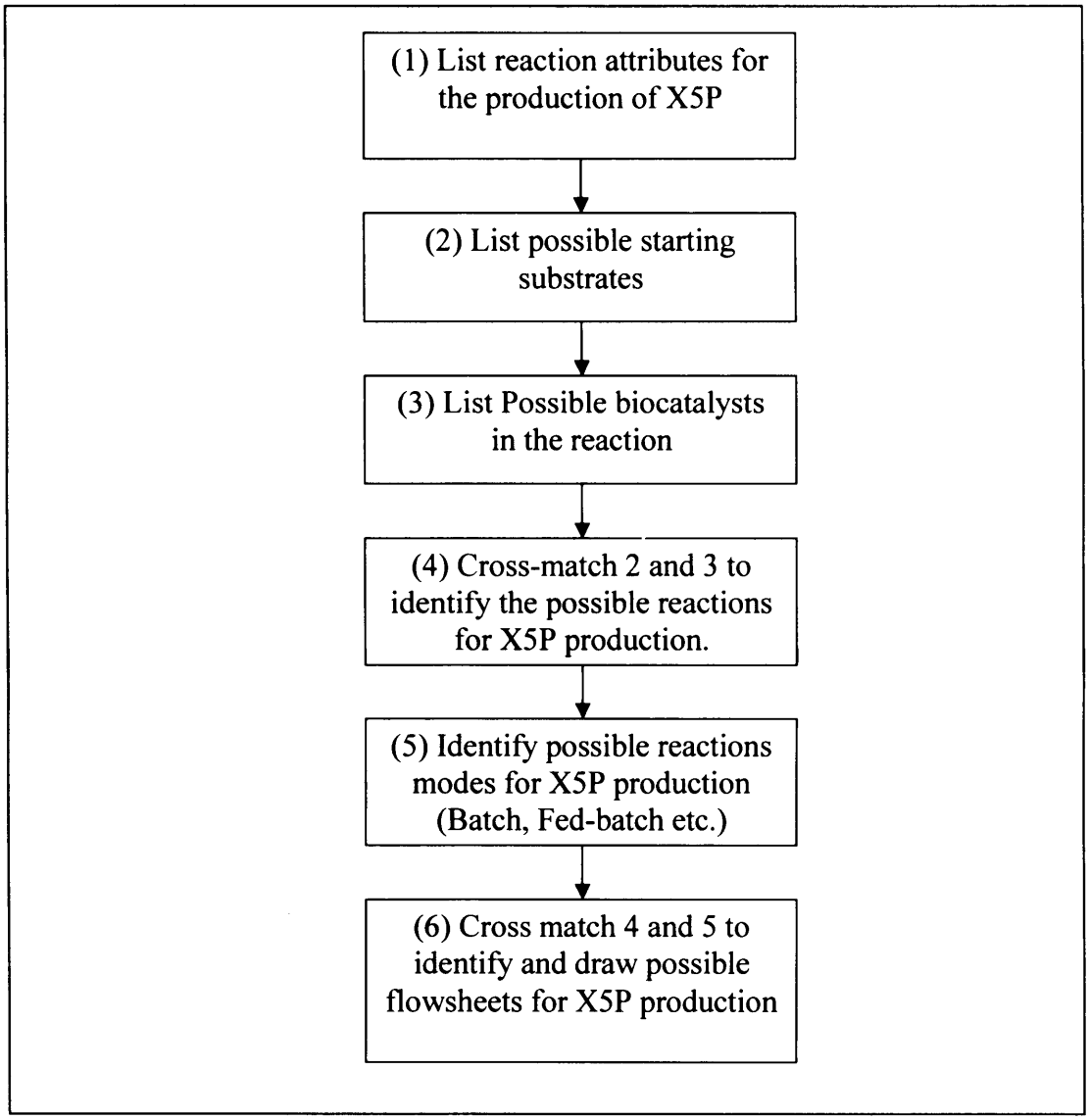


Figure 5.2 Logical step-by-step process synthesis for the X5P process.

5.2.1 Reaction attributes for the production of X5P

To design a set of processes for the X5P system it was deemed necessary to first lay down all important process components. The possible reaction attributes were noted with respect to starting substrate and the types of enzyme involved.

5.2.2 Possible starting substrates

G3P – Running reactions using G3P as a substrate showed good conversions. This substrate however is susceptible to degradation and has toxic effects on transketolase (Chapter 3). G3P is assumed to have higher value than DHAP.

DHAP – Using DHAP to produce X5P involves Tk and TPI. This reaction showed lower conversion. DHAP as a substrate was more stable and less toxic to Tk.

G3P and DHAP compromise - It was proposed in the characterisation stage that using the right ratio and mix of the two substrates would impact the reaction positively. It is possible to drive the equilibrium towards G3P and consequently improve X5P production.

5.2.3 Possible biocatalysts in the reaction

Here the varieties of biocatalysts possible for production were noted:

- Tkp and TPI – These two biocatalysts are necessary for the production of X5P from DHAP. When pure enzyme was used (Tkp) it was necessary to add TPI. Reaction did not reach completion without the presence of TPI to convert DHAP to G3P.

- Tkp – The use of this enzyme on its own is only possible in reactions starting with G3P.

- Tki – This enzyme source is the crude form of transketolase and contains within it other natural biocatalysts including TPI. This enzyme was sourced by fermentations at a lower cost than purchasing the pure form. Reactions

using this form of enzyme however have been shown to suffer from by-product production (Chapter 4)

- Tki and Tkp compromise – It was gathered by the experimentation in Chapters 3 and 4 that by adding Tkp to the crude Tki the ratio of Tk to TPI could be compromised to improve the reaction kinetics and thermodynamics.

5.2.4 Identifying the possible reactions for X5P production.

By cross matching the components involved it was easier to identify the possible reactions for X5P production. The possible reactions were then highlighted and the impossible reactions were eliminated (Table 5.1).

	G3P	DHAP	G3P and DHAP compromise
Tkp and TPI	●	○	○
Tkp	○	●	●
Tki	○	○	○
Tki and Tkp compromise	○	○	○

Table 5.1 The possible (○) and impossible (●) reaction attributes for the production of X5P.

From Table 5.1 it was possible to identify the possible components in the reactions for this model system. This was the first milestone in process synthesis. Without knowing the reactions it is impossible to draw accurate flowsheets (Shultz and Douglas, 2000).

5.2.5 List of possible reactions for X5P production

As a result of Table 5.1 the following reactions were possible.

1. G3P + Tkp → X5P
2. G3P + Tki → X5P
3. G3P + (Tki and Tkp compromise) → X5P

4. DHAP + (Tkp and TPI) → X5P
5. DHAP + Tki → X5P
6. DHAP + (Tki and Tkp compromise) → X5P

7. (G3P and DHAP compromise) + (Tkp and TPI) → X5P
8. (G3P and DHAP compromise) + Tki → X5P
9. (Tki and Tkp compromise) + (DHAP and G3P compromise) → X5P

From this list the next step was process synthesis and the drawing of process flowsheets. Developing this process design stage was influenced by the information and theories in conceptual design of chemical processes (Douglas, 1998), elementary principles of chemical processes (Felder and Rousseau, 1999) and process synthesis (Siirola, 1973).

5.3 Possible processes for X5P production

There are a variety of process methods for a multi-enzymatic preparation (Brinkmann *et al.*, 2001). At this stage the aim was to firstly illustrate the simplest forms of running the process as in a batch or closed system. Secondly looking at running the reactions in more precisely controlled systems (fed-batch) processes. Finally to devise more complex and non-integrated procedures to possibly to improve on the processes. The possible reactions identified earlier (Table 5.1) were cross-matched with three different modes of processes namely batch, fed-batch and non-integrated processes. This resulted in a variety of possible processes for the production of X5P. The resulting processes are shown in Table 5.2.

Possible reaction attributes	Batch	Fed-batch	Non integrated
G3P → X5P + Tkp	○ (Process 1)	○ (Process 2)	●
G3P → X5P + Tki	○ (Process 3)	○ (Process 4)	●
G3P → X5P + (Tki and Tkp compromise)	○ (Process 5)	○ (Process 6)	●
DHAP → X5P + (Tkp and TPI)	○ (Process 7)	○ (Process 8)	○ (Process 9)
DHAP → X5P +Tki	○ (Process 10)	○ (Process 11)	●
DHAP → X5P + (Tki and Tkp compromise)	○ (Process 12)	○ (Process 13)	●
(G3P and DHAP compromise) → X5P + (Tkp and TPI)	○ (Process 14)	○ (Process 15)	○ (Process 16)
(G3P and DHAP compromise) → X5P + Tki	○ (Process 17)	○ (Process 18)	●
(Tki and Tkp compromise) → X5P + (DHAP and G3P compromise)	○ (Process 19)	○ (Process 20)	●

Table 5.2 The possible (○) and impossible (●) X5P processes.

A variety of processes were highlighted (Table 5.2) many of which were not possible as non-integrated systems. This stage of process synthesis proved essential in understanding the various methods possible for the production of X5P. This was a logical method of identifying possible processes, useful in identifying possible processes in any multi-enzymatic system. It is important to identify as many processes as possible at this stage of process synthesis. Missing out on possible processes at an early stage was deemed to limit the analysis leading to disappointment later in process development if possible processes were to be

discovered at a late stage of analysis. Having identified the processes, they each must be analyzed or assessed further with regards to the type of process.

5.3.1 Batch processes

Classically the simplest method for running biotransformations is in a batch system. Batch systems form nearly half of reactor types used in industrial biotransformation (Straathof *et al.*, 2002). The production of X5P in processes 1, 3, 5, 7, 10, 12, 14, 17, and 19 (Table 5.2) was based on batch modes of operation. Each process designed to contain different starting substrates and enzyme mixes in a one-pot system. However these batch systems have limitations. These limitations are highlighted in detail in the characterization stages and lab-scale trials. As a consequence of enzyme impurity some processes suffer. Complete conversion is not achieved and the yields are often not satisfactory. To overcome these operational problems fed-batch and more complex processes were designed as improved batch systems, in effect allowing more control. Due to the experimentation in Chapter 3 and 4 actual data was already available on the attributes of the processes 1, 3, 7 and 10. The important information on these reactions was pooled together to aid process synthesis and analysis. Based on results these processes could be improved. For example in terms of biocatalyst. Increasing the level of transketolase fraction with respect to TPI should improve yield and productivity as well as product stream purity. Biocatalyst deactivation or substrate toxicity where too much substrate comes into contact with the enzyme at once is also a problem in batch processes. It was important to design systems where toxicity could be overcome or controlled also in order that higher product concentrations could be achieved. These were fed-batch systems.

5.3.2 Substrate feeding strategies

By feeding the substrates issues associated with toxicity would be overcome and higher product concentrations could be achieved. Processes 2, 4, 6, 8, 13, 15, 18 and 20 were devised primarily for this reason. The reactions run using a fed-batch system could suffer in terms of kinetics. The initial reaction rates may be limited by the feed rate of the substrate. The advantage of fed-batch systems was deemed to

be the greater control over the levels of substrate in the system. Using a fed batch process the components could be tightly controlled.

5.3.3 Non-integrated processes

Essentially a system such as the production of X5P can be considered as a multi-step production process. Therefore it may be possible to separate the enzymes normally involved in a multi-enzymatic systems. Process 9 and 16 were designed based on this idea. However they cannot be applied to every situation especially if the production in one step is dependent on the uptake by another step or if equilibrium issues are involved. An integrated system might be the only answer in the latter.

5.3.4 Compromised processes

More complex ideas manifested themselves in compromised processes 14, 15, 17, 18 19 and 20. These stemmed from the hypothesis that by compromising the substrate/biocatalyst concentrations and ratios it is possible to influence the process to be more efficient. This is very much possible with the availability of extensive data. Data is required on exactly how the reaction improves in the presence of different levels of both substrates and enzymes. At this stage the optimal substrate fraction data was not available perhaps resulting in the failure of these processes. Having understood the design of each mode of operation, flowsheets were constructed showing how each one would be adapted to industry. The designs at this stage of process development were kept rather basic and only sufficed as a generic picture of the process. It was assumed that for a quick indication of which processes were better the engineer would need to carry out assessments quickly and in the absence of detailed information. It was believed being able to make process decisions based on simple designs quickly was more important than adding detail to the flowsheets. The process flowsheets are illustrated in Tables 5.3, 5.4, 5.5 and 5.6. These drawings proved to be a fantastic tool in making a comparison and aiding analysis. Each of the process flowsheets was analysed carefully in terms of its advantages and disadvantages.

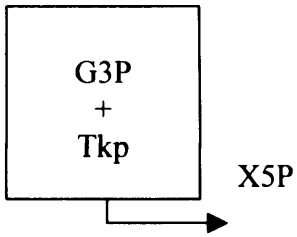
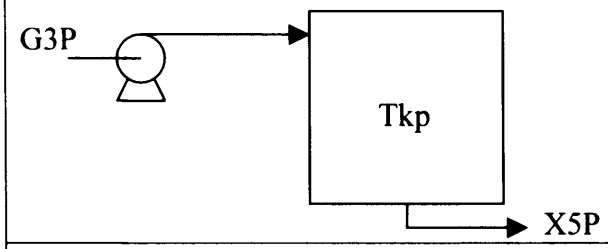
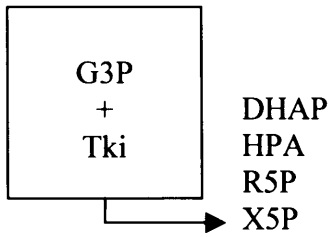
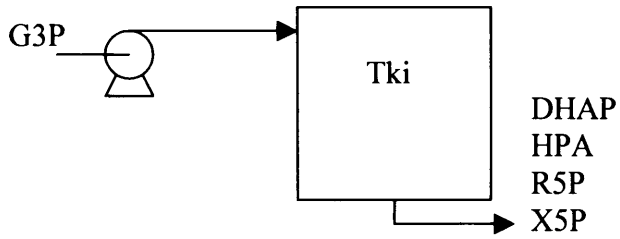
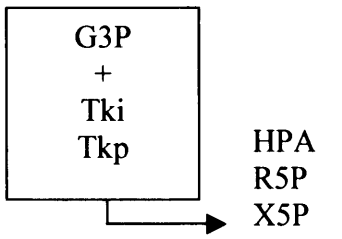
Process	Attributes
P 1: 	- Substrate inhibition. - Expensive process option. - Unstable substrate. - Good thermodynamics: all G3P convert to X5P. - High productivity.
P 2: 	- No substrate inhibition. - Lower STY than P1. - Reaction rate limited by G3P feed rate. - Clean product stream.
P 3: 	- Substrate inhibition. - TPI present in Tki resulting in the back conversion of G3P to DHAP and by-product R5P. - Poor thermodynamics as complete return on substrate not achieved.
P 4: 	- G3P expensive option for feed. - Some G3P converted back to DHAP. - Product stream contains substrate and by product. - TPI competes with Tk for G3P. Poor kinetics and thermodynamics.
P 5: 	- Substrate inhibition - Better conversion as TPI to Tk ratio kept to minimum with the addition of Tkp. - Some by-product formation.

Table 5.3 The diagrams / flowsheets for processes 1 - 5 with notes on their attributes.

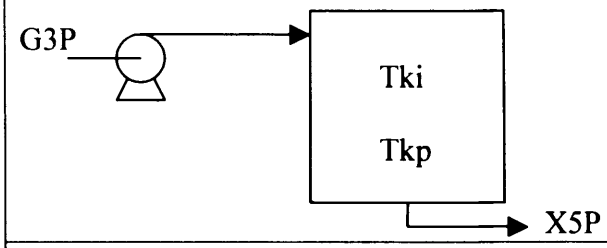
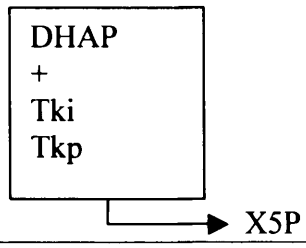
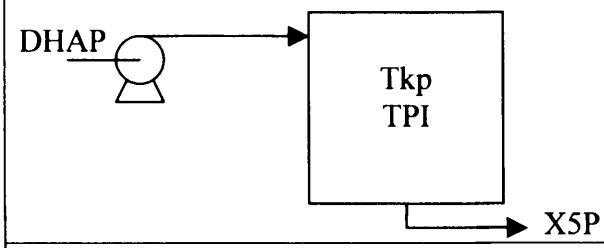
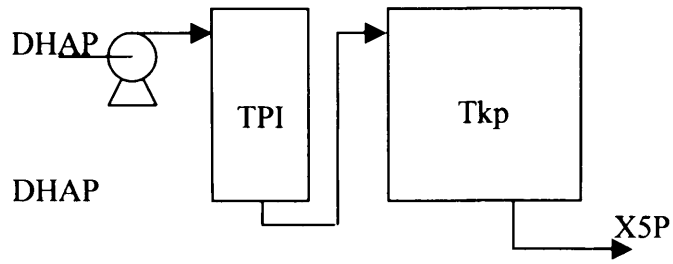
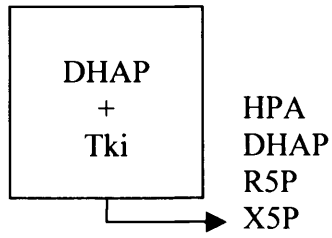
Process	Attributes
P6: 	- No substrate inhibition. - Kinetics limited by feed rate. - Better thermodynamics as G3P feed is used immediately. - Cleaner product stream.
P7: 	- DHAP substrate cheaper option. - TPI/Tk ratio favourable. - Results in better conversion. - Lower levels of substrate inhibition. - Cleaner product stream.
P8: 	- No substrate inhibition. - Lower STY than P7 due to feeding substrate slowing down reaction. - Improved thermodynamics on P7. - Kinetics suffers due to feed rate.
P9: 	- 2 stage process. - Complex to operate. - Firstly DHAP is converted to G3P using TPI. - Not all DHAP converted to G3P as TPI kinetics favour the production of DHAP.
P10: 	- TPI levels in Tki result in backward conversion of G3P to DHAP. - DHAP is converted to G3P by the TPI present in the Tki. - More economically viable option. -Product stream impure.

Table 5.4 The flowsheets / diagrams for processes 6 - 10 with notes on their attributes.

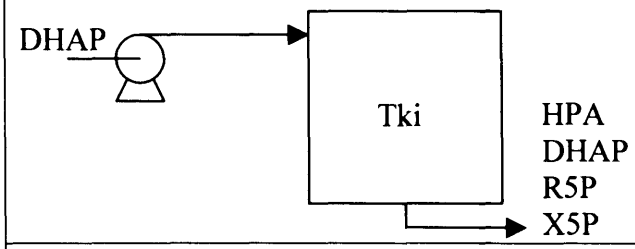
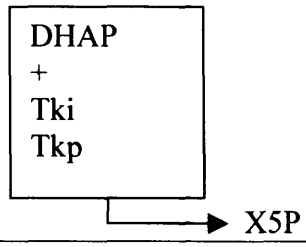
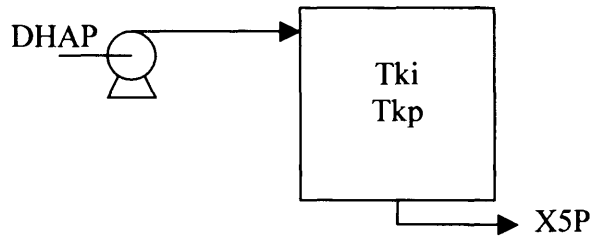
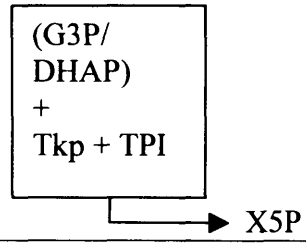
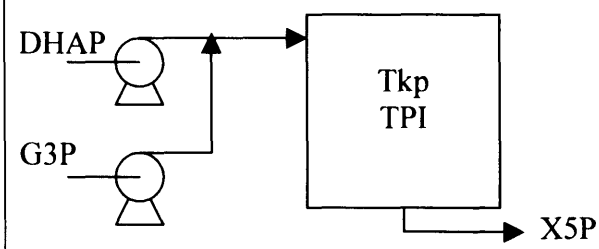
Process	Attributes
P11: 	- No substrate inhibition. - Not all of the DHAP converted to G3P consequently DHAP is expected in product stream. - Lower productivity than P10 due to limiting feed rate.
P12: 	- Reduced TPI/Tk ratio encourages better kinetics and thermodynamics. - Better productivity and STY. - Cleaner product stream.
P13: 	- Reduced TPI/Tk ratio. - Feeding DHAP reduces the back conversion of G3P to DHAP. - No substrate inhibition. - Better return on substrate. - Kinetics limited by feed rate. - Productivity limited by feed rate.
P14: 	- Substrate inhibition. - Both substrates used at compromised levels to drive reaction forward. - Difficult to calculate the right ratio. - Poor return on biocatalyst.
P15: 	- No substrate inhibition - Easier control of DHAP and G3P. - Presence of DHAP with G3P to prevent back conversion. - Improved kinetics and thermodynamics than P14. - High complexity.

Table 5.5 The flowsheets / diagrams for processes 11 - 15 with notes on their attributes.

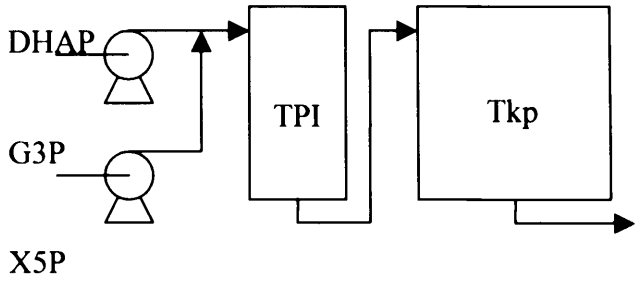
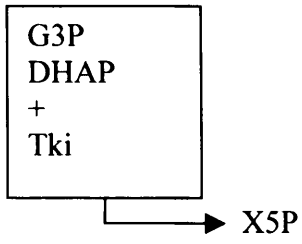
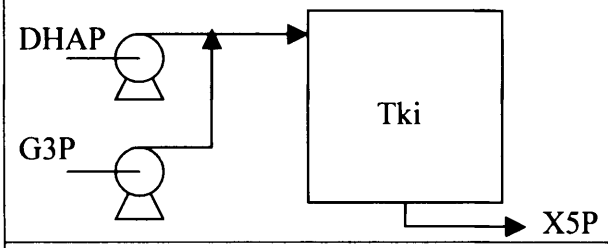
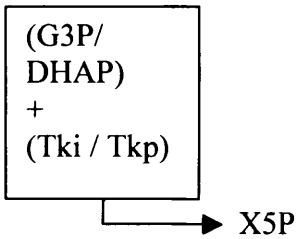
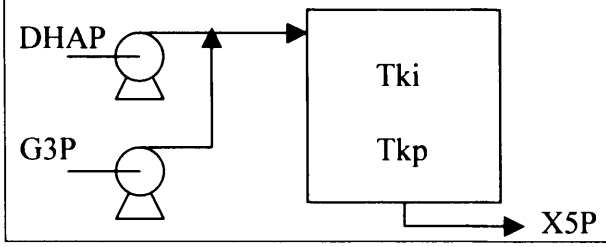
Process	Attributes
P16: 	- Controlled levels of substrates. - Improved kinetics on P9. - Poorer thermodynamics than P9. - Stops back conversion of G3P to DHAP.
P17: 	- Substrate inhibition. - Poor purity of product stream. - Improved kinetics on P10. - Poor thermodynamics due to the involvement of Tki.
P18: 	- No substrate inhibition. - Better control. - Lower productivity limited by feed rate. - Product purity suffers as Tki is used.
P19: 	- Substrate inhibition. - Improved thermodynamics on P17 - TPI/Tk ratio kept to minimum with the addition of Tkp.
P20: 	- No substrate inhibition. - Better kinetics and thermodynamics on P18. - TPI/Tk ratio more appropriate. - Better control. - Cleaner Product stream.

Table 5.6 The flowsheets / diagrams for processes 16 - 20 with notes on their attributes.

5.4 Logical process assessment and selection

It was aimed in this part of the chapter to devise a logical system for early process evaluation. All of what is learned about the multi-enzymatic X5P synthesis was gathered here to select the most promising process.

5.4.1 Process selection theory

Having synthesized 20 possible processes it was important to rapidly rule out the ones that were not desirable. In this Section calculations were made based on experimental data, logical and economic assumptions. The experimental data gathered on a few of these processes in Chapters 3 and 4 was immensely important here and aided the process analysis. The approach here was to score the different processes based on their attributes factoring in some economic data. These were referred to as the process costs scores. Having identified the cost estimates the processes were judged on their complexity. Process selection means that the processes are compared based on the cost scores and attributes such as thermodynamics, kinetics, productivity and purity (Straathof, 2003). Those scoring poorly were very quickly ruled out. Those processes with industrial potential were then compared and the most attractive options put forward as industrial candidates.

5.4.2 Process comparison

Some of the important data gathered earlier were used as indications and guidelines for estimating values for the unknown processes. The known information is represented in Table 5.7. The values from the actual biotransformations are helpful in estimating the attributes of the process flowsheets in Tables 5.3-5.6.

Reaction	X5P (mM)	Substrate (mM)	Yield [P]/[S]	Yield [P]/[E]	STY (g/L/hr)	Initial rate (g/L/hr)
DHAP → X5P +Tkp + TPI (P7)	17.13	20	0.89	2.04	1.08	9.29
G3P → X5P + Tkp (P1)	14.04	15	0.94	1.61	1.79	31.67
DHAP → X5P + Tkp + TPI (P7)	11.04	15	0.74	1.27	0.67	4.18
DHAP → X5P + Tki (P10)	13.40	20	0.67	1.54	0.76	3.76
G3P → X5P +Tki (P3)	15.20	20	0.76	1.75	0.86	4.24
DHAP → X5P + Tki (P10)	8.05	15	0.54	0.93	0.37	1.87
Fru 1,6 BP → X5P +Tkp + TPI (P21)	15.24	20	0.76	1.76	0.07	0.10

Table 5.7 Known information on some processes for X5P production (data gathered from experiments in Chapters 3 and 4).

The Table above was constructed from results shown in actual biotransformation profiles as seen in Chapter 3 and 4. The data for the fructose 1,6 bisphosphate reaction (P21) was also considered here. Even though process 21 had been eliminated in the characterization stages it was considered important to compare all possible processes together to obtain a more accurate picture for process development. It was important to observe how the process assessment procedure applies to process 21 to test the sensitivity of the model proposed here. Based on the data above and the attributes shown in Tables 5.3, 5.4, 5.5 and 5.6 kinetic and thermodynamic data was predicted for the rest of the unknown processes these results are shown in Table 5.8.

Process	Yield [P]/[S]	Yield [P]/[E]	Productivity ($g\ L^{-1}\ hr^{-1}$)	Initial rate ($g\ L^{-1}\ hr^{-1}$)
P 1	0.94	1.61	1.79	19.34
P 2	0.98	1.72	1.27	3.23
P 3	0.76	1.75	0.86	4.24
P 4	0.61	1.17	0.65	3.00
P 5	0.75	1.60	1.40	15.00
P 6	0.80	1.50	0.70	5.00
P 7	0.89	2.04	1.08	9.29
P 8	0.88	1.10	0.60	5.17
P 9	0.40	0.90	0.37	2.50
P 10	0.67	1.50	0.76	3.76
P 11	0.86	1.68	0.50	12.31
P 12	0.93	1.80	1.60	18.00
P 13	1.00	1.23	0.90	13.44
P 14	0.66	0.90	1.40	5.10
P 15	0.70	0.95	1.00	4.12
P 16	0.41	1.00	0.40	2.57
P 17	0.58	1.31	1.10	14.00
P 18	0.60	1.70	0.50	4.87
P 19	0.63	1.43	1.20	15.96
P 20	0.80	1.75	4.50	6.41
P 21	0.76	1.76	0.07	0.10

Table 5.8 Experimental data on known processes together with those predicted based on process attributes.

The kinetic and thermodynamic data on the processes provided much needed clues about their viability in industry (Wolff *et al.*, 1999). However attractive the reaction kinetics and thermodynamics are they do not guarantee industrial success. These data should not be compared without taking into account some important engineering economics. It is important not to base the process assessment model on direct economic assumptions. The aim here was to rule out processes early without using actual costs, which can be often inaccurate at the time of analysis.

5.5 The importance of value rather than actual costs

It is imperative that the process analysis or assessment model is not directly based on direct biocatalyst and substrate costs. The cost values available at this time are subject to alteration from the point of view of an industrialist. A company may have a cheap in-house source of one of the reaction components. Estimated costs may be used as examples for the process analysis model but a company might have a very large stock of pure transketolase or G3P reducing its value drastically. The process assessment and selection procedure should then be based more on the values of the components as perceived at the time of process development and not actual costs. The question here is how much more valuable is one component in comparison to the rest and not what the actual cost of the component is. By differentiating between the values of each component it became more distinguishable what the important factors were in the process. It was possible to make a set of logical assumptions. These assumptions served to simplify the economic analysis and make the process selection model more accessible to a whole range of multi-enzymatic reactions. The aim was to pull away from including actual costs in the process selection model and focus more on comparative value.

5.6 Simplifying the economic analysis for conceptual design

Simplifying the economic analysis required that the actual costs be taken out of the equation by replacing them with logical ratios. In order to make a comparison of a range of process flowsheets it is important first to make a series of assumptions regarding the values of the components involved in the processes (Shultz and Douglas, 2000). These economic assumptions were here based on a three step logical questioning. Figure 5.3 illustrates the step-by-step rational decision-making process proposed. Using this method leads to the subsequent valuation of all the components in the reaction.

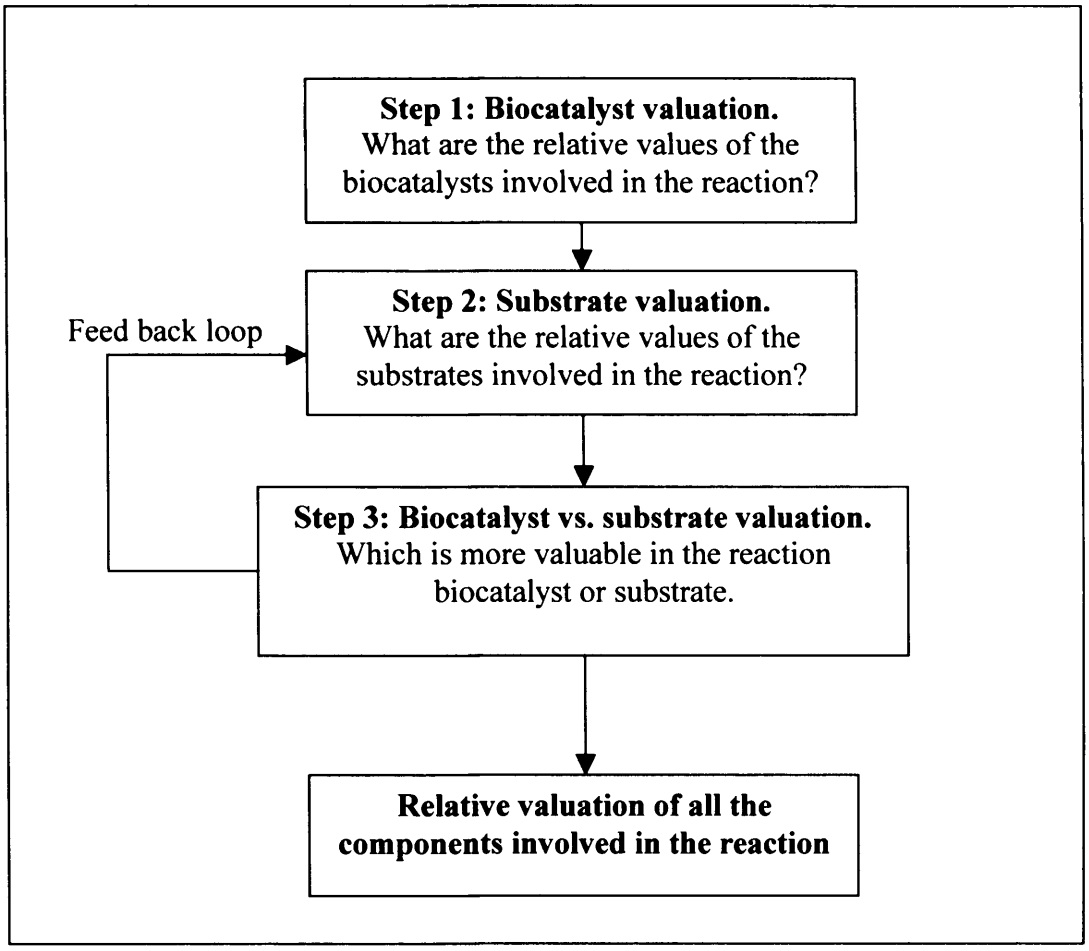


Figure 5.3 A step-by-step assumption process leading to the relative values of the components in a multi-enzymatic reaction.

It was decided that the above valuations (Figure 5.3) could be displayed simply in the form of ratios. Using the multi-enzymatic xylulose 5-phoshate synthesis as a model system the ratio assumptions were made (Section 5.6.1-5.6.4).

5.6.1 Step 1: Biocatalyst valuation.

Ratios were determined for the values of the enzymes involved. Purified transketolase is more valuable than impure transketolase and that they are both more valuable than the TPI needed in the reaction. Assuming that Tkp is approximately 7 times more expensive than Tki and that Tki is 16 times more expensive than TPI the following ratios were calculated. Tki is deemed to be 16 times more expensive than TPI based on the fact that in this study 50L fermentations were carried out to obtain Tki. TPI in contrast was purchased being available in large quantities on the market at the time of analysis. Also only a relatively small concentration was needed in the reaction vessel. The ratios were assumed based on three factors:

(1) Market availability.

(2) Concentration of component.

(3) Number of process steps needed to obtain the product.

However it must be noted that these ratios are assumptions applicable only at the time of analysis and may be altered easily at a later stage.

Values:	**Tkp**	**> Tki**	**> TPI**
Ratios:	**112**	**: 16**	**: 1**

5.6.2 Step 2: Substrate valuation.

Ratios were determined for the values of the substrates involved. G3P is the most valuable substrate as it degrades quickest, its availability is the key bottleneck to the reaction and it is more difficult to produce chemically than DHAP. DHAP is now widely available commercially and sourced relatively cheaply. HPA is the cheapest component as it is easily produced chemically and is more stable than DHAP and G3P. Assuming that G3P is approximately 10 times more valuable than DHAP and that DHAP is 1.5 times more valuable than HPA the following ratios were calculated.

Values:	**G3P**	**> DHAP**	**> HPA**
Ratios:	**16**	**: 1.5**	**: 1**

5.6.3 Step 3: Biocatalyst vs. substrate valuation.

Here it was important to decide whether the reaction economics were governed by the biocatalyst value or the substrate value. The value of the most expensive biocatalyst must be compared to the most expensive substrate. In the biocatalyst group and the substrate group the most valuable components determine whether the reaction economics biocatalyst or substrate dependent. Tkp has been identified as the most valuable biocatalyst (Step 1). Comparing its value to that of G3P (perceived to be the most valuable substrate) a ratio can be assumed.

Values:	**Biocatalyst (Tkp)**	**>**	**Substrate (G3P)**
Ratios:	**1.33**	**:**	**1**

5.6.4 The relative values of all the reaction components

Once the ratio in step 3 was distinguished it was necessary to go back to step 2 and alter the nominal value of the ratios. Not to change the ratios but to alter the numbers in accordance to the ratios in step 1 and step 3. The numbers in the ratios decided in step 2 are altered according to Tkp being 1.33 times more valuable than G3P. This resulted in the following substrate ratio.

Values:	**G3P**	**> DHAP**	**> HPA**
Ratios:	**84**	**: 8**	**: 5**

The ratio was not changed but the numbers were raised to compare them directly to the biocatalyst ratios. In this way it was possible to compare the values of all the components involved in the reactions. Table 5.9 shows the simplified values of all the components involved in the multi-enzymatic xylulose 5-phosphate production.

Values:	**Tkp**	**> G3P**	**> Tki**	**> DHAP**	**> HPA**	**> TPI**	
Ratios:	**112 :**	**84**	**: 16**	**: 8**	**: 5**	**: 1**	

Table 5.9 Simplified values assumed for the components involved in producing X5P, obtained using a step-by-step procedure illustrated in Figure 5.1.

It was consequently a simple task to make direct comparisons of the values of the reaction components. Table 5.10 shows a comparison of substrate values to that of enzymes and vice versa.

(Substrate/Enzyme)	*x*	(Enzyme/Substrate)	*n*
DHAP/Tkp	0.07	Tkp/DHAP	14.00
DHAP/Tki	0.5	Tki/DHAP	2.00
G3P/Tkp	0.75	Tkp/G3P	1.33
G3P/Tki	5.25	Tki/G3P	0.19
(G3P+DHAP)/Tkp	0.82	Tkp/(G3P+DHAP)	1.22
(G3P+DHAP)/Tki	5.75	Tki/(G3P+DHAP)	0.17

Table 5.10 Comparing substrate and enzyme values.

The value assumptions made in the step-by-step process described above served to provide process development as much economic information as possible about each process flowsheet synthesised in Section 5.3. Each of the decisions made above directly impacts the way in which any process is perceived. If the enzyme cost is higher than the substrates involved then the yield of product based on biocatalyst becomes crucial. This means that the reaction thermodynamics is important when biocatalyst cost is high. In the case of substrates being more valuable or expensive than the biocatalysts involved the yield of product based on substrate becomes more important. The reaction kinetics therefore becomes crucially important in the presence of a more expensive substrate. Table 5.11 illustrates these issues simply and relates them to the X5P process.

Enzyme value	**Enzyme value > Substrate value**
Depends on number of transketolase purification stages	Kinetics ($Y_{[P]/[E]}$) becomes important
Substrate value	**Substrate value > Enzyme value**
Depends on using G3P or DHAP	Thermodynamics ($Y_{[P]/[S]}$) becomes important

Table 5.11 The impact of biocatalyst and substrate values on the importance of thermodynamics and kinetics in the X5P processes.

5.7 Process assessment and selection

This is the final stage of evaluation in this chapter, largely concerned with highlighting the most viable and attractive processes for successful X5P production in industry.

5.7.1 Process assessment based on scores

As indicated in Table 5.12, thermodynamics and kinetics cannot be considered without taking into account the value of substrate and biocatalyst.

5.7.2 Process cost scores

The cost of a biocatalytic process can be divided into four distinct factors. Namely substrate cost, biocatalyst cost, bioreactor and downstream processing cost (Straathof, 2003). There are various ways of calculating real economic estimates for these as described in literature. It must be stressed that these calculations were in terms of scoring and ranking processes and consequently the numbers generated were of no particular units. A different approach may be taken in calculating these values but the scientific basis for the calculations of factors such as yield and productivity were universal ones. For the purposes of process selection it was considered logical that calculations should be kept as simple as possible so that they may be easily applicable a variety of processes. There was a danger of factoring in the attributes of the X5P example so much that the methods were rendered inaccessible by other similar reactions or problems. The equations proposed for calculating these cost scores have been given in full (Table 5.12). Some basic data was needed for calculating these equations. For example in each process it was important to determine the kinetics and thermodynamics at least as an estimate (as shown in Table 5.8).

Cost Score	Calculation
Thermodynamics: Yield [P]/[S] based on substrate cost	$Cost_S / (P/S)$
Kinetics: Yield [P]/[E]) based on enzyme cost	$Cost_E / (P/E)$
Bioreactor	$1 / (g/L/h)$
Downstream processing	$1 / (Purity * Concentration)$

Table 5.12 Mathematical equations for calculating each of the cost scores involved in process analysis.

5.7.3 Substrate cost score

The actual values of the components might not be directly reflected by their market price. It was important to use rational judgement as shown in Sections 5.5 and 5.6 in estimating substrate costs. If a chemical is difficult to produce or it is unstable and available in small quantities it may be deemed as a high value substrate. Raw material purity also determines its cost. The cost of the substrate is of major importance in the X5P reaction. There was a considerable difference between the cost of a gram of G3P and a gram of DHAP. G3P is only available in mg quantities at a time. It is also very unstable. DHAP on the other hand is now available relatively cheaply on the market in gram quantities. DHAP was considered the cheaper substrate here. More important than actual market prices was the economic ratios of the two substrates (Section 5.6). The actual market data on the substrates was similar to the values drawn between G3P and DHAP at this point in time. However they were subject to change therefore ratios were used instead (Section 5.6). Using these values it was possible to build up a picture of process cost with regards to the substrates and biocatalysts involved. These values alone did not provide a complete picture of process cost based on substrate. In a biocatalytic process the fraction of the reactant converted that ends up, as the desired product is key. Depending on whether the reaction shows acceptable thermodynamics it was possible to estimate this value. For some of these processes actual data was available. These were used to estimate values for the other processes. Table 5.8 shows the predictions that were made with regards to the thermodynamics and kinetics of each process. The thermodynamics cost score was the cost of the substrates involved in the reaction divided by the yield [P/S] estimated. Those process benefiting from good thermodynamics or in other terms good return of product from substrate gained a low cost score here. Those suffering from poor conversions gained a high cost score. The calculated substrate cost scores based on the thermodynamics of the 21 processes were calculated in Table 5.13.

Process	Yield [P]/[S]	Yield [P]/[E]	Initial rate ($g\ L^{-1}\ hr^{-1}$)	Enzyme Cost	Substrate Cost	Kinetics cost score	Thermodynamics Cost score
P 1	0.94	1.61	19	112	89	4.25	95.12
P 2	0.98	1.72	3	112	89	17.97	91.10
P 3	0.76	1.75	4	16	89	14.87	117.11
P 4	0.61	1.17	3	16	89	21.33	146.62
P 5	0.75	1.60	15	128	89	5.36	118.67
P 6	0.80	1.50	5	128	89	13.69	111.25
P 7	0.89	2.04	9	113	13	1.15	14.66
P 8	0.88	1.10	5	113	13	2.07	14.79
P 9	0.40	0.90	3	113	13	3.82	32.50
P 10	0.67	1.50	4	16	13	2.47	19.40
P 11	0.86	1.68	12	16	13	0.93	15.17
P 12	0.93	1.80	18	128	13	0.66	13.99
P 13	1.00	1.23	13	128	13	0.89	13.00
P 14	0.66	0.90	5	113	97	16.17	147.64
P 15	0.70	0.95	4	113	97	19.13	139.57
P 16	0.41	1.00	3	113	97	27.17	234.87
P 17	0.58	1.31	14	16	97	6.34	168.40
P 18	0.60	1.70	5	16	97	14.76	161.67
P 19	0.63	1.43	16	128	97	5.58	153.00
P 20	0.80	1.75	6	128	97	11.89	121.25
P 21	0.76	1.76	0.1	113	17	9.16	22.31

Table 5.13 Calculating thermodynamics and kinetics cost scores.

5.7.4 Biocatalyst cost score

The cost of the biocatalyst involved in a bioprocess was shown to be estimated accurately using the ratios in Table 5.9. Transketolase has been over expressed in *E. coli* and therefore its manufacturing costs have been drastically reduced. There was the option of using a semi-purified transketolase extract from fermentation. This type of transketolase was considered the cheapest source. Pure transketolase available on the market was more expensive in comparison. Again to achieve more accurate cost data the whole production process in terms of the enzyme needed to be considered, from the fermentation down to purification.

The biocatalyst cost in a process does not solely depend on whether a cheap source is used or not. To accurately estimate the cost of the biocatalyst in a bioprocess it is important to include information about its kinetics in the reaction. There are some kinetic data available from the few processes run previously. The rest of the processes were estimated by way of kinetics [P/E]. The cost of the enzymes involved in a process divided by the yield [P/E] provided a kinetic cost score, which was particular to each process. Better kinetics in a process resulted in a low biocatalyst cost score whereas poor kinetics meant a high cost score. Table 5.13 also shows all the calculated kinetics cost scores.

5.7.5 Bioreactor cost

It was desirable to apply high substrate or biocatalyst concentration to a reactor as to reduce its volume (increase process intensity). This means that a smaller bioreactor can be used, which directly reduces cost at the same time reducing bioprocess plant space needed. In the same way it was deemed that a process with better productivity (g/L/h) would also reduce bioreactor cost. Using the estimated productivity of processes it was possible to score processes based on their bioreactor cost. From the equation in Table 5.13 if a reaction had the advantage of high productivity it had a low bioreactor cost score. If it suffers form poor productivity it scores highly in terms of bioreactor cost. Bioreactor cost scores were calculated for the 21 processes and have been listed in Table 5.14.

Process	Productivity (g L-1 hr-1)	Purity score	Bioreactor cost score	DSP cost score
P 1	1.8	9.0	0.6	0.11
P 2	1.3	10.0	0.8	0.10
P 3	0.9	5.0	1.2	0.20
P 4	0.7	4.0	1.5	0.25
P 5	1.4	6.0	0.7	0.17
P 6	0.7	6.5	1.4	0.15
P 7	1.1	7.0	0.9	0.14
P 8	0.6	8.0	1.7	0.13
P 9	0.4	5.0	2.7	0.20
P 10	0.8	4.5	1.3	0.22
P 11	0.5	5.0	2.0	0.20
P 12	1.6	7.0	0.6	0.14
P 13	0.9	6.0	1.1	0.17
P 14	1.4	5.5	0.7	0.18
P 15	1.0	6.5	1.0	0.15
P 16	0.4	3.2	2.5	0.31
P 17	1.1	4.5	0.9	0.22
P 18	0.5	3.7	2.0	0.27
P 19	1.2	4.5	0.8	0.22
P 20	0.5	5.5	2.2	0.18
P 21	0.1	7.0	13.5	0.14

Table 5.14 Calculating the bioreactor and DSP cost scores.

5.7.6 Downstream processing

It was considered best to obtain a high product concentration to simplify the downstream processing (Chapter 4). This also meant that the product stream should also be as pure as possible. Consequently it was also considered important to factor in the purity of the product stream. In the case of X5P production an impure product stream makes it necessary to incorporate anion and cation exchange steps into the downstream processing (Chapter 4). If the mass balance of the process is closed and there are no by-products it can be predicted that the downstream processing will be relatively cheap. The purity of the product stream of each process was given a score. Residual substrate in the product stream or impure enzyme resulted in poor purity score (1.00). Complete conversion or the use of pure enzyme was given a high purity score (10.00). The equation for DSP cost score in Table 5.13 indicates that if a process is estimated to have high product concentration and purity score then that process will score low. Processes with low product concentration and poor purity will score highly on DSP cost. The values for each process are also shown in Table 5.14.

5.7.7 Process complexity

Operational problems were accounted for here as a guideline for selecting processes. Termed as process complexity it was possible to score flowsheets or process designs depending on how problematic they were deemed to be on an industrial scale. It was decided that for the production of X5P there are four main issues contributing to its complexity. Substrate toxicity or inhibition of transketolase, substrate stability in reaction environment, by-product production and scalability were all accounted for in this part of process analysis.

5.7.7.1 Substrate inhibition /toxicity

Molecular toxicity means that at high concentrations the reaction stops or becomes too slow. Much of the enzyme is inactivated which in turn means that this effect must be counteracted with the addition of more biocatalyst. Table 5.16 contains details on the level of substrate inhibition by way of loss of transketolase activity (%) for each process. 30mM G3P causes 15 % loss of activity whereas 30mM DHAP causes 8 % biocatalyst deactivation. There are also toxicity issues in terms

of the product X5P. It was deemed as a general assumption this affects all the processes equally. Therefore product inhibition was not factored into the complexity here. In this case in situ product removal was not considered as the substrates are of similar chemical property and not only would this change the working volume it would also reduce biocatalyst concentration effecting the conversions negatively.

5.7.7.2 Substrate stability

G3P and DHAP are the raw materials in the X5P production process. To understand the complexity of using either substrate some information about the nature of the raw materials must be included. The first assumption was that purity is at the same standard in both. However the same cannot be said for their stability. The stability of the components in the reaction is a major key in determining its complexity. The loss of substrate due to instability is undesirable in a process. Here DHAP was more stable as a substrate than G3P. DHAP suffers only 4.14% loss in a bioreactor in comparison to 30.10% loss of G3P at typical reaction conditions (pH 7.0, 25 °C).

5.7.7.3 By-product formation

It is particularly necessary to account for any side reactions that might take place. A trace amount of a by-product produced in a laboratory experiment may build up to very high levels at industrial scale. Side reactions occur when crude enzyme extract is used. This affects the purity of the product and increases the need for more intensive downstream processing. By-products may in industry bring on the need to synthesise a separation system adding to the complexity. They are also an indication that the substrates in the system are not being used efficiently. Crude transketolase extract allows side reactions to occur with the HPA present in the system. These by-products may also alter the kinetics of the reaction. All processes containing impure transketolase score high due to by-product formation (Chapter 4). Those containing pure enzyme score a minimal 10 points those including Tki score a high 30 points. Overlooking side reactions is a common mistake and almost always leads to paying large economic penalties.

5.7.7.4 Scalability and bioreactor design

Industrial economic analysis should take into account the scalability of a process. This issue is mainly concerned with whether a designed process can easily and comparably be adapted to large scale. Fed batch operations are rather complex in their operation and design. There are relatively smaller numbers of biocatalytic operations in industry that are run based on a fed-batch strategy. Online analysis and control is rather expensive and difficult. Multi-step processes also suffer due to their complexity. Here batch processes were deemed to be the least complex in terms of scalability and bioreactor design (10 points). Fed-batch systems were deemed to be more complex to scale up (30 points) and Non-integrated processes the most complex as they require scale-up calculations for more than one reaction step (30 points). All the complexity calculations are shown in Table 5.15 for the processes.

Process	G3P toxicity	DHAP toxicity	G3P stability	DHAP stability	Substrate toxicity score	Substrate stability score	By product production	Scalability (bioreactor design)	Total complexity score
P 1	15	0	30	0	15	30	10	10	65
P 2	0	0	30	0	0	30	10	30	70
P 3	15	0	30	0	15	30	30	10	85
P 4	0	0	30	0	0	30	30	30	90
P 5	15	0	30	0	15	30	30	10	85
P 6	0	0	30	0	0	30	30	30	90
P 7	0	8	0	4	8	4	10	10	32
P 8	0	0	0	4	0	4	10	30	44
P 9	0	0	0	4	0	4	10	30	44
P 10	0	8	0	4	8	4	30	10	52
P 11	0	0	0	4	0	4	30	30	64
P 12	0	8	0	4	8	4	30	10	52
P 13	0	0	0	4	0	4	30	30	64
P 14	15	8	30	4	23	34	10	10	78
P 15	0	0	30	4	0	34	10	30	74
P 16	0	0	30	4	0	34	10	30	74
P 17	15	8	30	4	23	34	30	10	98
P 18	0	0	30	4	0	34	30	30	94
P 19	15	8	30	4	23	34	30	10	98
P 20	0	0	30	4	0	34	30	30	94

Table 5.15 Calculating the complexity scores of the processes.

5.8 Results

The work in this part of the investigation suggested a logical approach to synthesising a series of processes for a model multi-enzymatic system. The processes were then analysed with great care and attention given to their attributes. The aims were to design processes to analyse the flowsheets and to select the ideal process to be put forward to industry. In terms of process synthesis a step-by-step approach was taken starting with listing the components involved in the reaction. This approach proved very useful in aiding the process designer in the creative stages of process development. The important components and attributes were gathered and cross-matched in tables to build relevant processes. These processes included batch, fed-batch and non-integrated modes of operation. The step-by-step process synthesis suggested in this chapter give the process designer the confidence to move towards process analysis. It was very unlikely that a possible process design was forgotten or missed out due to this logical progression. Finding out that a critical process was not considered in the later stages of process analysis could prove disastrous. The process engineer must backtrack to the very beginning and re-conduct the analysis, which in turn prolongs the time for development. The work in this chapter described a quick and easy method of analysing processes in such a way that noticing a new possible process at later stages would not hinder the investigation.

In total 20 processes were proposed for the production of X5P using a logical process synthesis strategy. If additional processes were to be suggested they would just simply be put through the subsequent analytical process. The processes were first considered in terms of their possible yield and productivity. This enabled a comparison based on the kinetics and thermodynamics of each process. With the aid of a simple substrate and enzyme cost estimation it was possible to score the processes based on thermodynamics and kinetics. Scores were also given for bioreactor costs, downstream processing costs and complexity of the processes. Using the scores given in Sections 5.7 it was subsequently possible to select the best process to put forward for industrial use of scale-up.

5.8.1 Process selection

The scores calculated in the assessment section (Section 5.7) were here used to compare the processes. Again a logical approach was taken where the processes were compared firstly based on kinetics and thermodynamics cost scores where those processes suffering from poor kinetics and thermodynamics and high substrate and enzyme costs were eliminated. Secondly the remaining processes were compared with regards to DSP and bioreactor cost scores and finally in terms of complexity. The process selection logic is described in Figure 5.4.

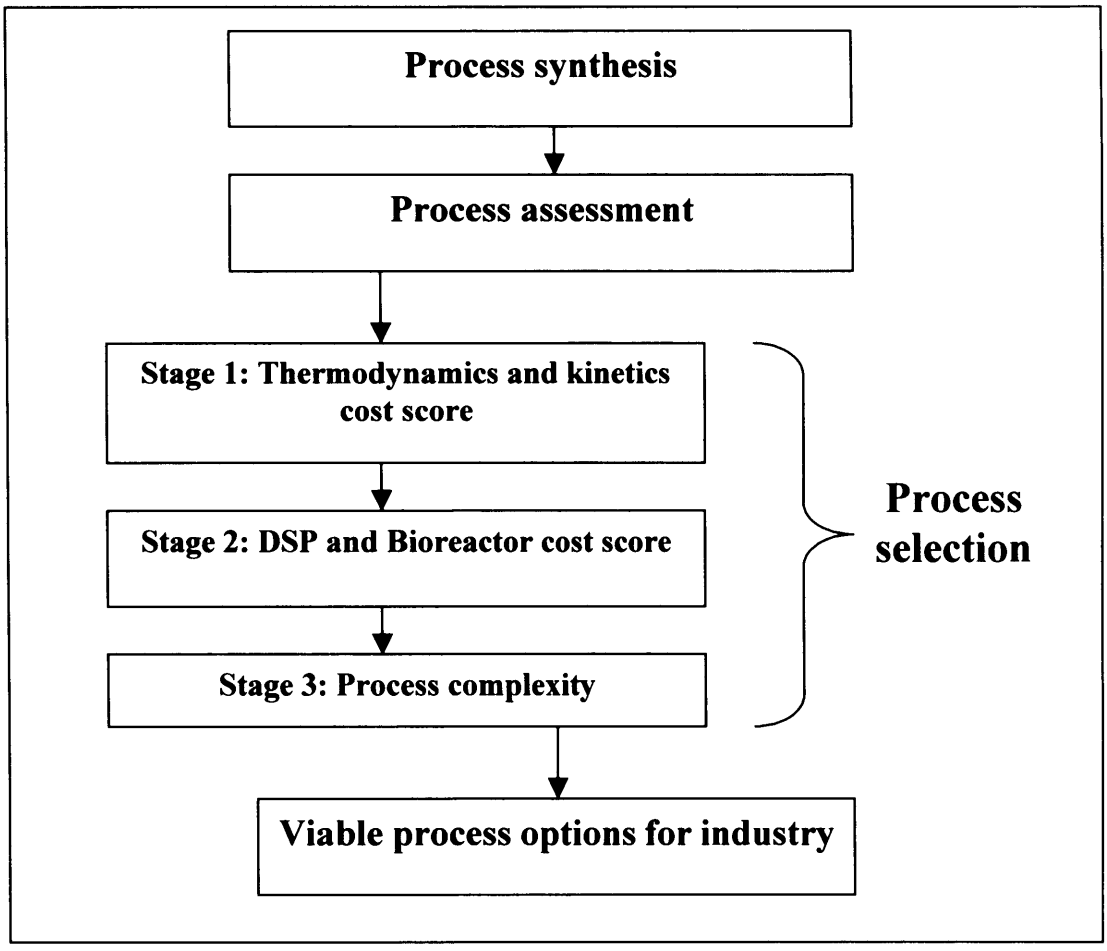


Figure 5.4 Logical process selection.

5.8.2 Stage 1: Thermodynamics and kinetics cost score

Comparing the thermodynamic yield (P/S) cost score with that of the kinetic yield (P/E) cost score proved very important in the initial stages of process selection. These are scores that provide great insight into the workings of each process. There was a clear indication on whether a process is cost heavy with regards to substrate or biocatalyst. Particularly looking at the processes in Figure 5.5 it is clear where the processes stand in comparison to each other. It is possible to place boundaries on what scores are deemed acceptable and what cost scores are too high to be profitable. The boundaries placed in the case of X5P production are also shown in Figure 5.5 as diagonal lines. These boundaries may be based on economical limits placed by an industrial economics analyst.

The importance of this stage is the possibility of completely ruling out certain processes. Those processes scoring very highly on either axis will not be considered as viable or operable due to inefficiency. A very important question that needed to be addressed here was the relativity of the two scores. The sensitivity of this scoring model was considered a major issue in validating this selection process. The sensitivity of this model was analysed by looking at changing certain values in Table 5.13. In the case of a cheap substrate source the cost of G3P was halved. This is very likely in many cases where companies can produce certain components relatively cheaply in-house. It is apparent that some processes move across boundaries as a result of cheap enzyme or substrate (Figure 5.6). This proved a very important sensitivity analysis indicating that some processes as possibly successful in case of cheap substrate or biocatalyst. These processes must be investigated further to allow for any errors made in the economic estimations. A selection of processes deemed to be promising at this stage were carried forward to the next stage of process selection.

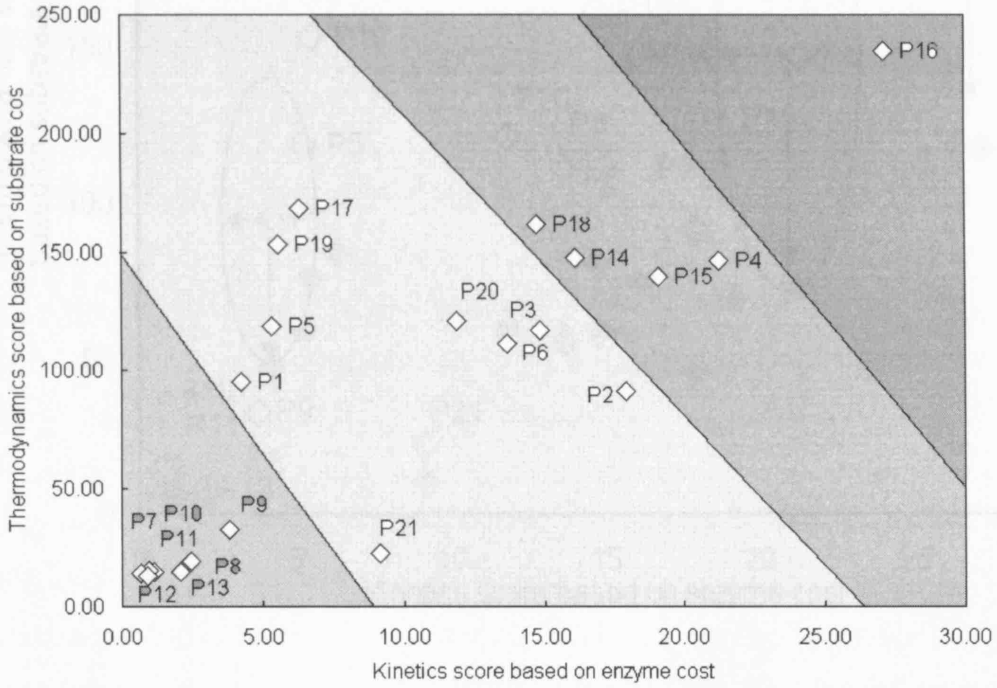


Figure 5.5 Assessing a set of processes based on kinetics scores (based on enzyme value) and thermodynamics scores (based on substrate value).

From the above figure it was clear that processes 9, 10, 7, 11, 8, 12 and 13 were strong contender as the model process in industry. Applying the sensitivity analysis showed that the processes moved vertically based on substrate value (Figure 5.6) or horizontally when biocatalyst value was altered (not shown on figure due to overcrowding). This meant that each process moved within a radius around itself based on changes made to the costs or depending on any optimisation.

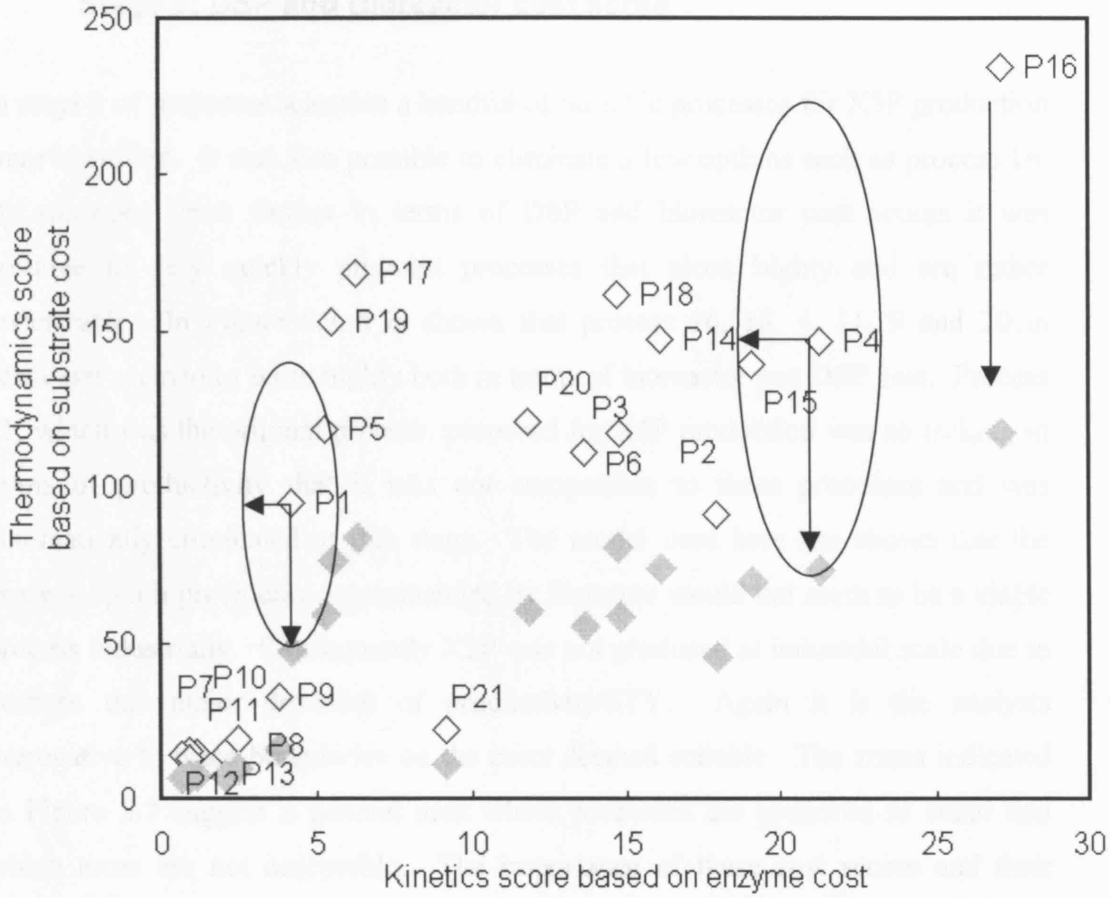


Figure 5.6 Sensitivity analysis of the assessment technique. As a result of halving substrate values processes move position in the chart area. The new positions (◆) indicate the outer limits of the plane of optimization shown by ellipses for P1 and P4. The arrows indicate the radius of the planes.

These planes of optimisation can be represented as closed circles/ellipses where it is possible to see processes overlapping. This was considered a powerful analysis later in process development where it would show which processes have potential for optimisation and which don't. For example in Figure 5.6 it is possible to optimise P1 to join the group of P7. It is clear that this is not possible with P5.

5.8.3 Stage 2: DSP and Bioreactor cost score

In stage 1 of processes selection a handful of possible processes for X5P production were identified. It was also possible to eliminate a few options such as process 16. By analysing them further in terms of DSP and bioreactor cost scores it was possible to very quickly pinpoint processes that score highly and are rather undesirable. In Figure 5.7 it is shown that process 16, 18, 4, 11, 9 and 20 in particular seemed to score highly both in terms of bioreactor and DSP cost. Process 21, which was the original process, proposed for X5P production was so lacking in terms of productivity that it was not comparable to these processes and was automatically eliminated at this stage. The model used here has shown that the process option previously recommended by literature would not seem to be a viable process industrially. Consequently X5P was not produced at industrial scale due to perhaps this major downfall of productivity/STY. Again it is the analysts prerogative to place boundaries on the costs deemed suitable. The zones indicated in Figure 5.7 suggest a general area where processes are preferred to stand and which areas are not acceptable. The importance of these cost scores and their relationship with each other is case specific. We can see that processes 1 and 2 stand very closely in terms of their scores. But are they in actuality as easily operable as each other? The third and final stage of this analysis was concerned with taking into account the complexity of the processes.

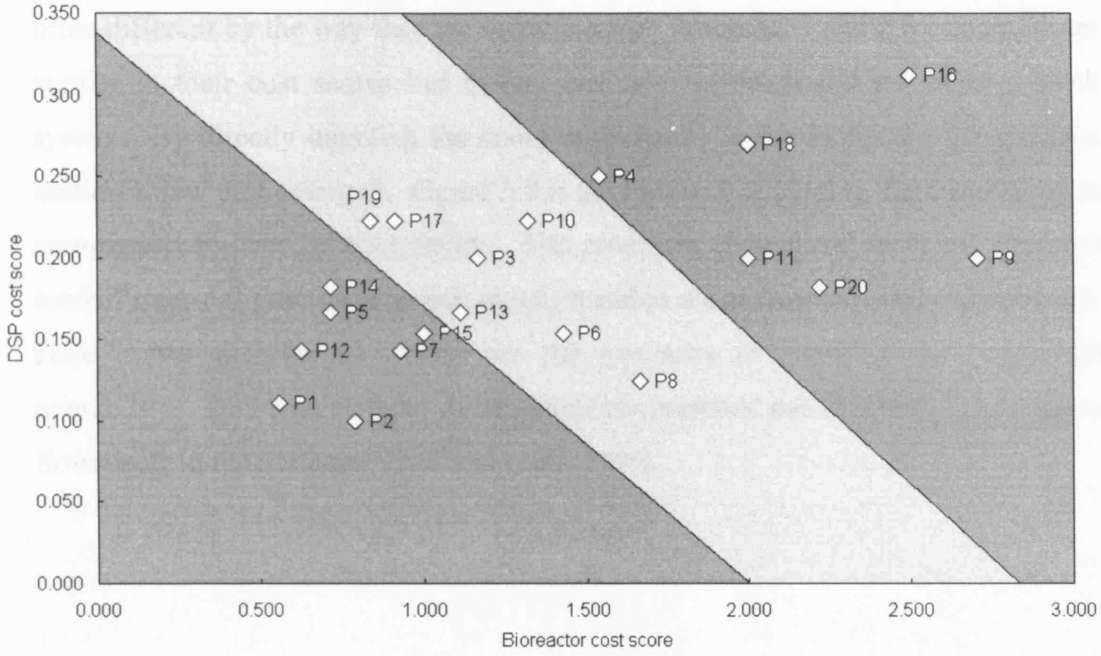


Figure 5.7 Assessing processes based on their bioreactor cost scores and their DSP cost scores.

5.8.4 Stage 3: Process complexity

By this stage of process selection the results and the scores had successfully identified the non-viable or poor process designs. These were in order, P4, P9, P11, P16, P18, P20 and P21. The strength of using this system of process selection was deemed to be this early detection of unattractive processes. It was still necessary to decide between the remaining processes the most industrially applicable ones. Figure 5.7 showed that processes P1, P2, P5, P7, P12, P15, and P14 were possible contenders for the final model process. They all survived the rigorous stage 1 and 2 of process selection. However a process that scores well in terms of cost is not necessarily the easiest to implement in industry. Some of the processes seem

closely related in terms of cost scores in Figures 5.5 and 5.7. In actuality they are often different by the way they are implemented. Processes 1 and 2 for example are similar in their cost scores but in fact one is a fed-batch and the other a batch system. By directly injecting the complexity scores to the scores for the previous section a new plot emerged. Figure 5.8 is the best way of placing these processes in comparison by way of complexity. The processes short-listed from the first and second stages of process selection clearly stand in a three dimensional environment. Here it was possible to clearly see the processes in terms of their industrial operability. This type of three dimensional environment has been used to compare flowsheets in the literature (Steffens *et al.*, 1999)

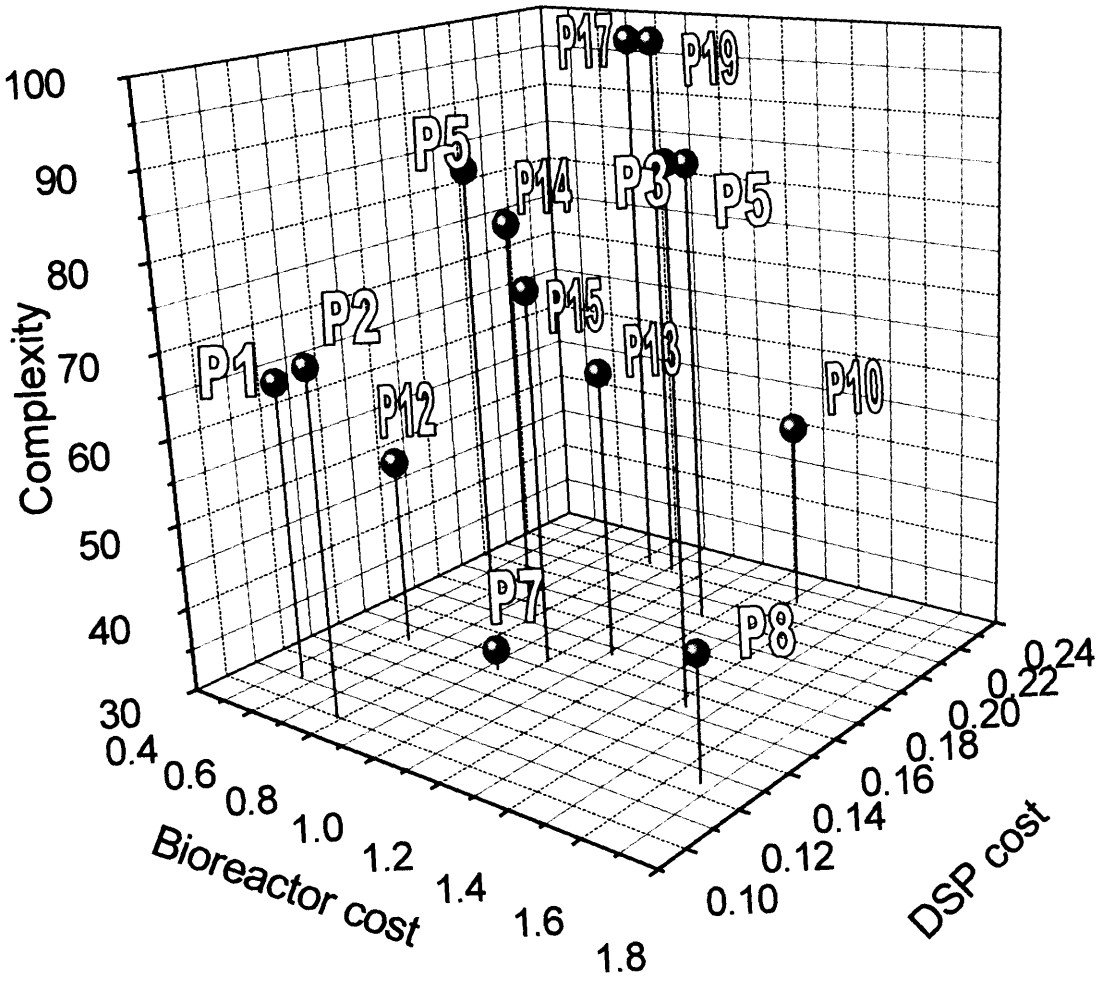


Figure 5.8 Selecting processes by considering their complexity together with bioreactor and DSP cost scores.

Processes P1, P2, P5, P7, P12, P14 and P15 in Figures 5.5 and 5.7 scored well in terms of their cost but it is clear that their complexity sets them apart from each other.

5.9 Discussion

5.9.1 Dealing with cost score inaccuracies

Cost estimates however well informed are likely to be inaccurate at the time of process selection. It is important to note that in this example the cost of TPP was not factored into the equations for calculating scores. The presence of co-factors was necessary for all the processes designed. For simplicity the value of the cofactors added was assumed to be equal for all processes. Similarly most of the components used in small concentrations in the processes were not included in the costing procedure. However not accounting for components such as TPP may later cause inaccuracies in this process-screening model. What may seem like a small amount in the laboratory will be costly at large-scale.

It is also important to note that when producing TK at the fermentation stage, thiamine is added to the broth so that there is enough TPP in the overexpressed cells. Although this aided the production of holo-transketolase further addition of TPP and pre-incubation was still necessary in achieving activity (Brocklebank *et al.*, 1999). The inactive apo-enzyme must bind with enough TPP to produce the active holo form. Further research will prove useful in producing more TK in the holo form therefore eradicating the need for TPP addition and pre-incubation time. TPP can therefore eventually be left out of the process costing with little consequences on accuracy.

Figure 5.6 showed how sensitive the current costings were to change and therefore how sensitive the whole process selection model is. It is also possible to pull out cost as a factor and just represent the process scores in terms of the ratio of enzyme / substrate (n). The n values were factored into the analysis to aid the process analysis in terms of the value of biocatalyst /substrate. The results are illustrated in Figure 5.9.

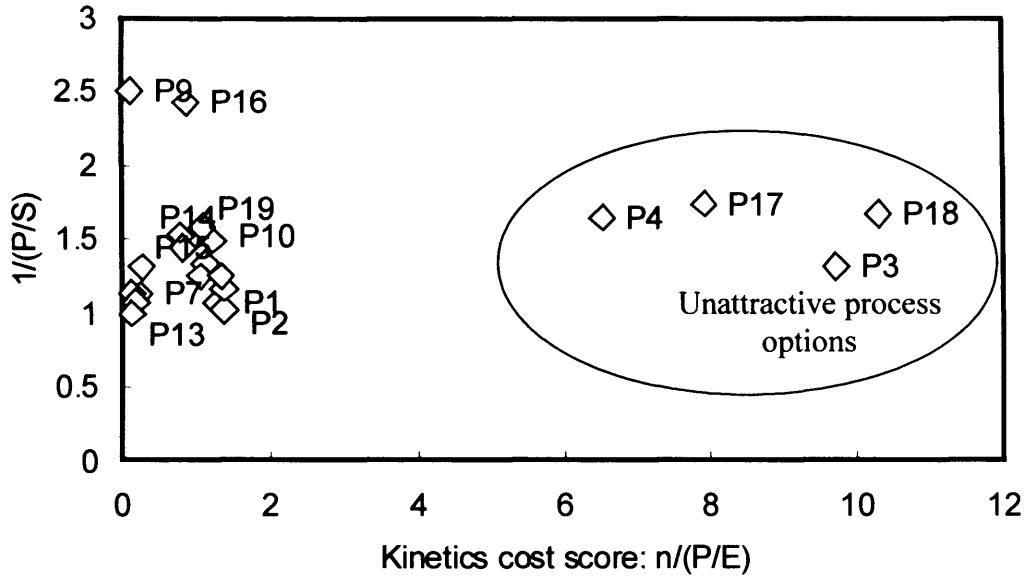


Figure 5.9 Assessing processes in terms of kinetics $n/_{(P/E)}$ and thermodynamics ($1/_{P/S}$) by taking cost out of the equation and taking into consideration the value of enzyme / value of substrate (n).

It is possible to again define areas within this space where we deem processes unattractive. The optimal area in this space is where complexity is low and cost scores minimal.

The process selection model proposed here has successfully eliminated process options P4, P11, P9, P18 and P16 at a very early stage (Figure 5.5). In the first stage it was gathered that these processes suffer from kinetic and thermodynamic problems as well as cost issues. Processes P7, P1, P10, P3 and P21 had been carried in the laboratory previously (Chapters 3 and 4). The results indicated in Table 5.7 are from actual experiments. This processes passed the first stage of process analysis. It seemed that the cost issues outweighed the benefits of process options P4, P11, P9, P18 and P16 for the production of X5P. These processes were simply not economically viable and it is likely that they would not be taken up in industry. For these processes the product price is lower than production costs rendering them non viable. Process P11 failed in stage one and P10 seems to be the least promising in later stages. These are both processes including impure enzyme (Tki). In these cases it was clear to see that the unfavorable equilibrium position

caused by residual TPI present in Tki is limiting the feasibility of non-integrated process.

During process selection process P21 (Starting with Fru1,6BP) which was ruled out in the characterisation stages was so unfavourable that it did not appear in the same vicinity of the other processes (therefore not shown in Figure 5.5) and was subsequently eliminated in the first stage of process selection. The processes remaining after the elimination rounds were P1, P2, P7, P8 and P12. These processes stand a good chance of successful scale-up and industrial application.

5.10 Summary

By constructing this process selection model real engineering has been applied to a biocatalytic process. The production of X5P was very problematic due to the involvement of more than one enzyme. A process where yields on substrate were low. The only option for achieving better yields was to use a very expensive substrate (G3P) and pure enzyme (transketolase). Process synthesis provided an array of possible options.

In part one of this investigation process synthesis was carried out by asking a set of questions about the enzymatic reaction at hand such as:

1) What are the process attributes in terms of kinetics and thermodynamics?

2) What are the substrate or raw material attributes in terms of toxicity and stability?

Based on these scientific facts engineering answers were provided in the form of a set of flowsheets or processes.

In the second part of this study these engineering suggestions were put to the test using a process selection model. The selection model drew on scientific and engineering knowledge and was injected with some economic estimation. Each of the processes synthesized was put under scrutiny. The processes were ranked and graded. This analytical model ruled out many of the processes suggested by process synthesis and pinpointed a few that should be carried out for the production of X5P. The logical progression suggested here is a promising method in shortening the development time of biocatalytic processes. From a large list of possibilities it was possible to identify which ones were worthy of investigating further in the

laboratory. Bioprocesses have rarely had the opportunity to be developed in this way. The future success of biocatalytic processes lies in developing more logical systems like this one. If they are to be readily accepted in the industry as chemical processes enzymatic reactions must be developed with the industry in mind. There is a definite trend of design and optimisation methods for bioprocesses emerging from literature. These ideas must be applied and further developed for different reactions. Every enzymatic system can take advantage of the methods suggested here. The benefits could help the current trend in gathering guidelines and methodologies for developing industrial biotransformations.

Figure 5.10 summarises the methods used in this chapter and indicates which processes where eliminated at which stage of process selection.

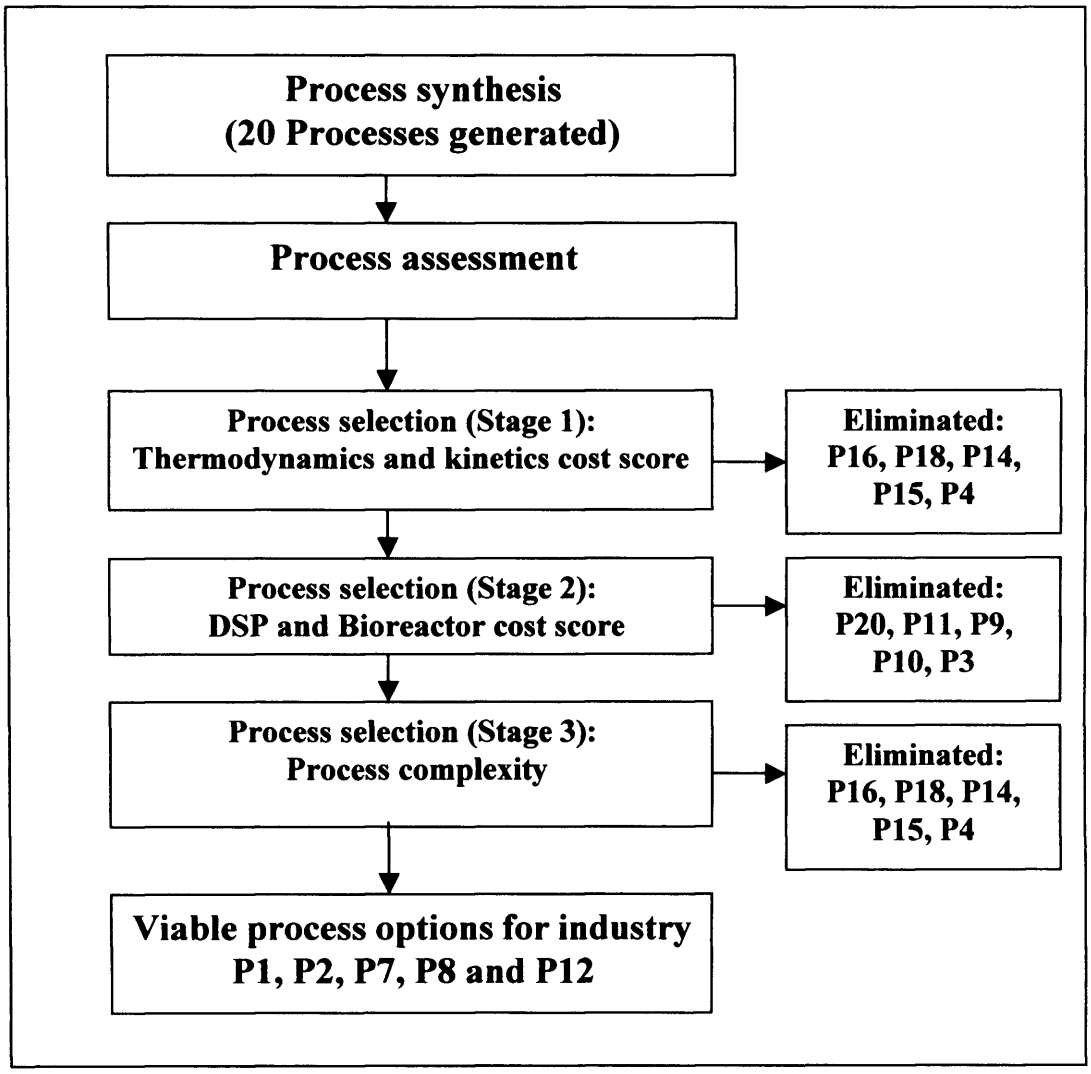


Figure 5.10 Process synthesis, assessment and elimination of unfavorable processes by logical process selection.

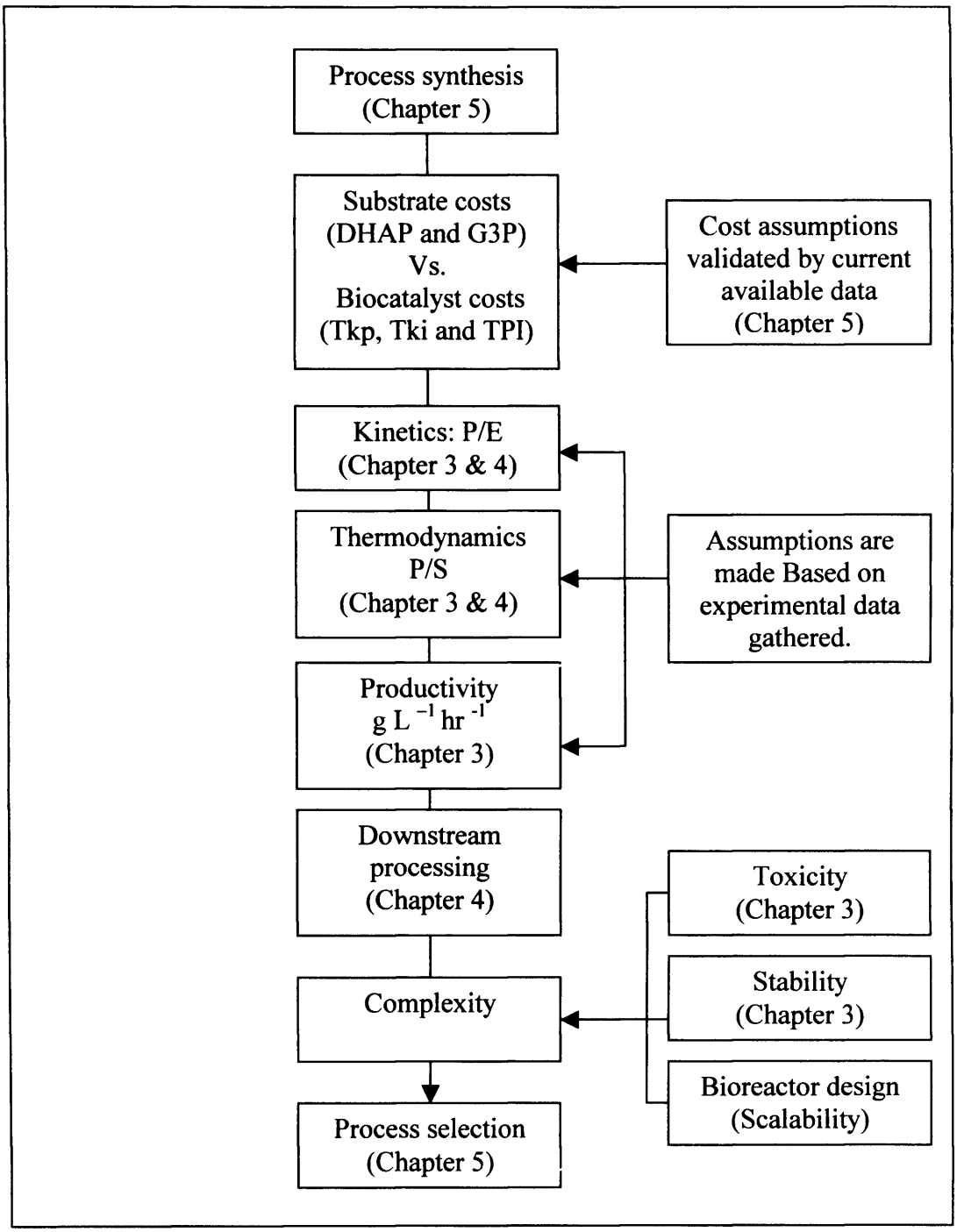


Figure 5.11 The logical progression from process synthesis through to process assessment and selection.

6 General Discussion

In this research a systematic approach was taken to improve a multi-enzymatic model system. In a one pot procedure, fructose 1,6 bisphosphate aldolase initially catalyses the retro-aldol cleavage of D-fructose 1,6 bisphosphate to give a mixture of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate, which can be equilibriated by the use of triosephosphate isomerase (TPI). D-glyceraldehyde 3-phosphate then couples with HPA in the reaction mediated by *E.coli* Tk to give D-xylulose 5-phosphate.

This model system was important in developing Tk as a biocatalyst for asymmetric C-C bond synthesis. An enzyme, which has been over-expressed and is now truly available in substantial quantities if required. Much effort has gone into defining the problems associated with the operating the reaction at scale and in certain cases genetic solutions have been identified (Turner, 2000). Transketolase is being increasingly used to perform regio- and enantioselective reactions in chemoenzymatic synthesis and therefore has immense synthetic potential (Sprenger and Pohl, 1998).

The lack of design data and no clear precedent in many instances mean that new biotransformations are developed on a case-by case basis leading to long development times. Therefore reducing the time to scale up of biotransformations is an important aim of the underpinning engineering in order to exploit the full power of enzyme-based catalysis (Woodley and Mitra, 1996).

This research was largely based on the design philosophy that exploits a structured decision making procedure (Woodley and Lilly, 1994), which enables design decisions to be made at the earliest possible opportunity (initially eliminating poor designs and later targeting good ones). This procedure involved first the characterisation of the system to identify the constraints. Secondly the scale-up and understanding issues such as the reactor environment and product recovery enabled defining of process options. This enabled the selection, integration and operation and eventual evaluation of limited options.

6.1 Characterisation

In the characterisation stages the results showed that the model reaction suffered constraints. The substrates were found to be pH sensitive as well as the product. Operating windows (Woodley and Titchener-Hooker, 1996) proved to be vital in understanding the results. In literature there are examples where the windows plotted relate enzyme fraction to pH (Hogan and Woodley, 1999).

The calculations of detailed kinetic parameters and a kinetic model for the reaction are not shown in this research. Although the biotransformation profiles could have been used for this it was deemed necessary to focus on the characteristics of the reactions in terms of process yield, productivity and efficiency using initial rates, product concentration and yield on substrate. There are numerous examples in literature, which refer to the modelling of the transketolase reaction (Vasic-Racki *et al.*, 2003). The analysis of the kinetics in multi-enzyme systems has also been described, Methods of MEKA where calculation of Vmax/Km values lead to determination of substrate concentrations have been found highly successful in glycolipid biosynthesis (Bieberich and Yu, 1999). The kinetics of the Δ6-desaturase have also been analysed using computer modelling to calculate Km and Vmax in a multi-enzyme system (Ivanetich *et al.*, 1996). Although these methods would have been highly beneficial in obtaining more information about the model reaction in the absence of large quantities of substrate it was deemed more reasonable to only obtain data vital for improving the final reaction. It was essential to obtain information specifically relevant to process design and development. Gathering extensive data on the chemistry of the model reaction would have been scientifically valuable but would have taken up much time and resources. In the end having the detailed parameters and kinetics of the reaction was not recognised to ensure process success. These techniques will however be essential in the latter stages of process design to potentially improve and optimise process options. This would be a strategy for the improvement of process design changing constraints (Woodley and Lilly, 1996).

The kinetics of over expressed transketolase form *E. coli* JM 107/pQR 700 have also been shown in terms of the glycolaldehyde reaction (Gyamerah and Willetts,

1997). The same methodologies can be applied to the X5P system in the future for further in-depth characterisation purposes.

6.2 Lab-scale design

When a bioconversion process is developed, it is desirable to apply a high substrate concentration to the reactor (reduce volume and increase efficiency) and to obtain a high product concentration to simplify downstream processing (Wolff *et al.*, 1999). In this study when scaling up the processes increasing substrate concentration was considered but was not applied. In this study the processes run at lab-scale were all of relatively low molarity (less than 20 mM). A reason for this was the high value-low concentration of the substrates at hand. Aside the cost issues it was considered more valuable to run a larger number of biotransformations to gain more insight into the process issues. Also the characterisation stages showed that at lower concentrations inhibitory effects of the product and the toxicity of the substrates would be minimised.

The downfall of running the lab-scale reactions at low concentration was that they fell in a zone where the thermodynamics and the kinetics of the G3P and DHAP reactions were not similar (zone A, Figure 3.22 and 3.23). In this zone the G3P reaction had the upper hand in terms of kinetics and thermodynamics. The DHAP reaction needed to be improved to compare with the G3P attributes (chapter 5). Running reactions at higher concentrations would have meant that the DHAP reaction would have been on par with the G3P reaction (zone C Figure 3.22) or benefit from better kinetics (Zone D Figure 3.22).

The overall outcome of the results in Chapter 4 leads to a comparison between two processes. One option is to run with crude enzyme and the other is to run with pure enzyme. As stated in the methods Section 2.2.4 the crude source in this case was susceptible to loss of activity. This loss of activity was probably due to protease activity (Gorbach, 1980). Therefore it is important to also note that the presence of PMSF and benzamidine as protease inhibitors in the purified enzyme preparation reduced the loss of activity in transketolase. This is an important advantage of using the purified transketolase source, which was not considered in the tradeoffs in Section 4.4.4. Experiments should be carried out in future on the inhibition of

transketolase together with SDS gels to identify the exact level of expression and the purity of the enzyme source used. Also the inhibitory effects of the product will need to be addressed perhaps by adopting ISPR strategies (Chauhan *et al.*, 1997).

6.3 Process synthesis, analysis and selection

Logical decomposition of biotransformation process design pointed to identifying appropriate process options after determinations of the constraints. This was considered to involve the use of rules and heuristics (Dervakos *et al.*, 1995). In this final stage of the study it was important to design a set of processes and to identify the most profitable and industrially attractive processes rather than those that produced maximum yield (Shultz and Douglas, 2000)

Logical process synthesis lead to the design of 20 possible options for X5P production. The flowsheets were basic and preliminary and lacked detail. It was noted that to encourage speed in process development the processes needed to be designed very simply at first with only the essential streams added. These could then be used to judge the processes based on their attributes such as kinetics, thermodynamics and productivity.

This type of multi-criteria process synthesis has been useful in generating sustainable economic bioprocesses (Steffens *et al.*, 1999) where it enables subsequent selection of processes based on factors including economics and productivity (STY). The process flowsheets were scored based on thermodynamics, kinetics, substrate and biocatalyst cost, productivity and downstream processing. Using a sequential selection procedure the unattractive processes were eliminated (Chapter 5). The final group of processes were compared based on a graphical representation using three criteria (Complexity, bioreactor cost score and DSP cost score). In summary a list of processes were identified for the production of X5P.

The method used here eliminated poor choices from further consideration and generated attractive alternatives without solving the optimisation problem (Mahajanam *et al.*, 2001). By changing the value of parameters such as substrate value it was clear that each process could be considered as an island (Siirola, 1996). It would subsequently be possible to optimise for example cleaner processes which might not be the cheapest options or the most scalable to compete with more industrially attractive options. The logical process synthesis and assessment

developed in this thesis gained much from other sections of the study as described in Figure 6.1.

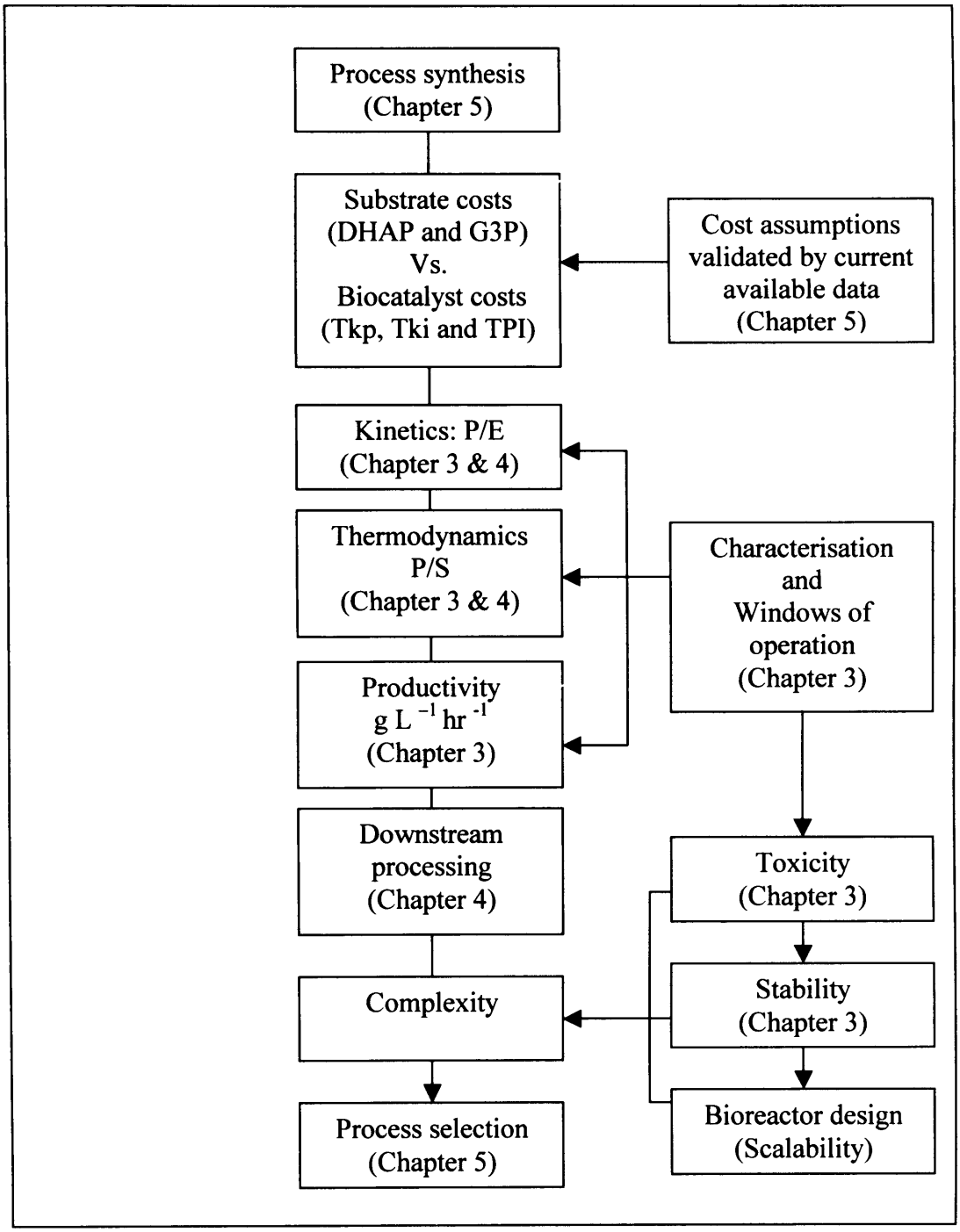


Figure 6.1 The logical progression from process synthesis through to process assessment and selection.

6.4 Investigating a biotransformation with multiple enzymes

In this thesis the multi-enzymatic system was first considered by characterising it's components and running preliminary biotransformations. This proved to be beneficial as some possible processes for X5P preparation were eliminated. Figure 1.6 also shows how important the characterisation and windows of operation were in the X5P process development. For specialty chemicals the time-to-market becomes a dominant consideration, which implies a minimum number of experiments and basing a design on incomplete data (Shultz and Douglas, 2000). The characterisation stage and the windows of operation in Chapter 3 provided much data and knowledge for the subsequent process selection stages. Process synthesis and assessment would have been impossible or highly inaccurate was it not for the results obtained in Chapter 3 and 4. The work in this thesis highlights the importance of bridging the gap between windows of operation (Woodley and Titchener-Hooker, 1996) and process synthesis and assessment (Siirola *et al.*, 1973, 1996 and Douglas, 1998), which results in a reliable, rapid, and comprehensive process development tool. There are now a number of tools available enabling a more systematic approach to process development (Lye *et al.*, 2002). Three such tools are namely:

1- Process modelling, the use of engineering and cost models for rapid process analysis and specification of development targets, which was applied in this investigation.
2- Micro scale processing techniques, which could help develop the type of analysis by allowing a massive number of processes to be tested at small scale very rapidly (e.g. process synthesis and assessment of 92 microwell options rather than 20).
3- Bioinformatics to aid biocatalyst identification and accelerated directed evolution, which could from the results gained here customise the cells biocatalyst levels and improve the Tki properties intrinsically.

The methods developed here could be used together with the above tools to further advance process modelling and development.

6.6 Applications

The methods developed here can be applied to other multi-enzyme systems to great effect. For example in the synthesis of the unnatural sugar 4-deoxy-D-fructose 6-phosphate. Firstly, (R,S)-1,1-diethoxy-3,4,epoxybutane is resolved by treatment with an epoxide hydrolase from *Aspergillus niger* to give recovered (S)-epoxide in 30% yield and >98% enantiomeric excess. The epoxide is converted to the (S)-aldehyde by treatment with inorganic phosphate followed by acidic hydrolysis. The aldehyde is then reacted with L-erythrulose in the presence of transketolase to generate 4-deoxy-D-fructose 6-phosphate in 52 % yield. (Uerard *et al.*, 1999).

Fuctose 1,6 bisphosphate aldolase (EC 4.1.2.13) from rabbit muscle is the aldolase most widely employed for preparative synthesis owing to its commercial availability (Fessner and Helaine, 2001). It is the most frequently used enzyme in enzyme catalysed aldol reactions (Seoane, 2000). A large portion of compounds produced using biotransformation processes are carbohydrates (Straathof *et al.*, 2002). Further investigation into the fructose aldolase system could be possible with the use of improved analytical techniques. Online nuclear magnetic resonance (NMR) monitoring of biocatalytic reactions is of growing interest (Hansjorg and Becker, 2000). The use of NMR detection coupled with HPLC offers the advantage of providing structural information about the components separated on the column (Krunker *et al.*, 2004). This type of complete analysis would prove revolutionary to the understanding of multi-enzymatic systems.

7 Conclusions and future work

7.1 Overall conclusions

In this work the multi-enzymatic production of X5P was investigated. Firstly the reaction was characterised. Based on the guidelines lab-scale productions and purifications were developed. The results highlighted important issues to consider in the development of the process on an industrial level. A strategic process synthesis, analysis and selection procedure was developed. The techniques helped present viable process options for industrial and commercial use. The most important results from this investigation are summarised below:

The multi-enzymatic synthesis of X5P was characterised by conducting a series of preliminary experiments. Preparation of X5P from Fru1,6BP was unsatisfactory due to the low productivity (0.07 g/L/hr) and the long lag phase (6 hours) experienced.

Transketolase from *E.coli* JM107 pQR711, suffers from substrate inhibition and some product inhibition. The product X5P was shown to cause a toxic effect of greater than 20% above 30mM concentrations. The substrates were inhibitory to the process. In particular G3P was shown to cause inhibition above acceptable levels at concentrations higher than 80mM

The stability of the substrates was studied at a wide range of values. DHAP was a more stable substrate than G3P, which showed a 35 % loss in comparison to DHAP losing 25 % after 6 hours (pH 6.5, 30mM).

Preparation of X5P from G3P and DHAP was carried out at lab-scale. Using G3P gave the best results in terms of product concentration (3.2 g/L), kinetics ($Y_{[P]/[E]}$ 1.61), thermodynamics ($Y_{[P]/[S]}$ 0.94) and productivity (1.79 g/L/hr) in

comparison to DHAP product concentration (2.5 g/L), kinetics ($Y_{[P]/[E]}$ 1.27), thermodynamics ($Y_{[P]/[S]}$ 0.73) and productivity (0.67 g/L/hr).

Purity of biocatalyst was shown to be of key importance in the multi-enzymatic system. Using Tki in the DHAP reaction, the product concentration was 43 % below that of the G3P reaction.

Purification of X5P indicated by-product formation where Tki was used and R5P was detected and identified by NMR.

A set of 20 processes was synthesised for the preparation of X5P from G3P and DHAP using Tki and Tkp.

Using a logical process assessment and selection unfavourable processes were eliminated. Using this technique, 5 processes were short listed for the production of X5P at large scale or for industrial application. The process selection method proved sensitive to changes and a useful tool in process development.

7.2 Future work

This work largely focused on the interplay of DHAP and G3P together with Tk and TPI in the system. There is no reason why it should not be possible to identify the exact ratios of aldolase, TPI and Tk in a one-pot system. Further research can result in the production of X5P form fructose 1,6 bisphosphate but with the same productivities and yields as those achieved with DHAP and G3P. Previous attempts probably suffered inefficiencies due to lack of information on the level of substrates and biocatalyst.

Further experiments are needed on the levels of substrate. This time including fructose 1,6 bisphosphate with G3P and DHAP. This would mean an increased number of experiments. Depending on the concentrations tested this will be very time consuming and expensive at the scales used in this work. The use of microwell systems would be very advantageous where a large number of biotransformations can be run at various substrate concentrations. These reactions require pH control and although there have been some developments in downsizing pH control for microwells it would be reasonable to run buffered systems. Buffering small-scale reactions with low concentration Tris-HCl (10-50 mM) should suffice for the reaction and at the same time minimise interference.

Further research needs to be carried out to extend the work undertaken here to fully characterise fed-batch biotransformations involving multiple enzymes. Due to the high value – low concentration of components such as G3P this is more feasible at micro scale. Using an accurate small-scale autotitrator or automated multiprobe substrates can be fed into small reaction volumes (2-4mL) and the resulting X5P tested using HPLC. As only a minute amount is needed for detection running reactions in this manner is feasible.

- More data is needed on TPI in terms of its sensitivity to the substrates. The toxic or inhibitory effects of the substrates on TPI were not investigated in this research.

- Research need to be extended to validate the process synthesis, analysis and selection procedure developed in Chapter 5. It needs to be established whether the systematic approach is applicable to other processes. Further research can add accuracy to the scoring system. This is possible with tracking how issues such as scalability, costs and productivity are viewed in industry. The weights and importance of these issues is often not equal and this must be factored into the scoring system. Waste production is possibly an issue that is likely to have a great impact of process selection as more companies and governing bodies are increasingly concerned with the environmental impact of processes.

Appendix I: HPLC analysis

Method File Name : c:\peaknet\method\jobin2.met
Date Time Collected : 05/09/03 16:28:58
System Operator : Jobin Shaeri

Vial ID :
Injection Volume : 1.00
Injection Number : 1

Peak Information : All Peaks

Peak #	Component Name	Retention Time	Amount	Peak Area	Peak Height
1		2.97	0.00	36994179	798310
2		6.15	0.00	2489029	186882
3		6.63	0.00	33991883	2373111
4		8.48	0.00	23351682	1570494
5		9.75	0.00	1419339	123615

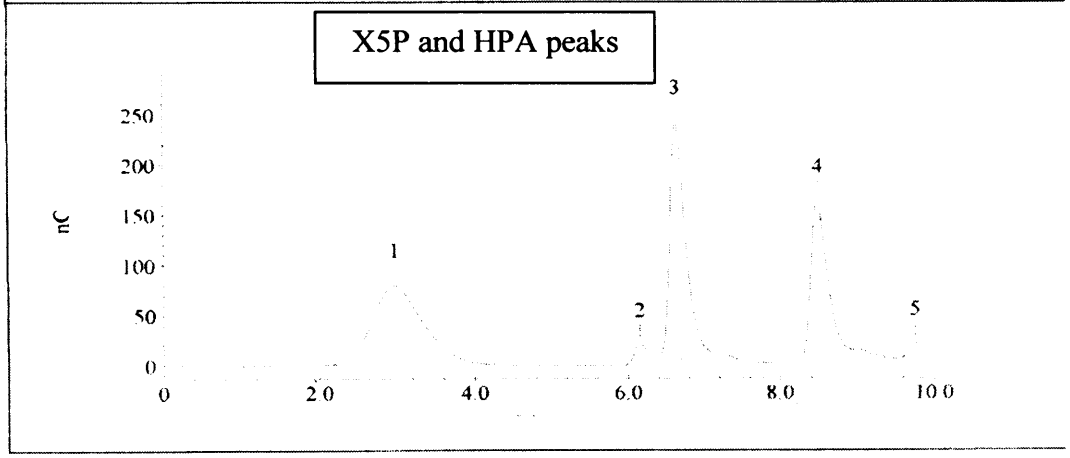


Figure I.1 X5P (peak 3) and HPA (peak 4) as detected with HPLC method described in Chapter 2. Retention times were 6.63 (X5P) and 8.48 (HPA).

Method File Name : c:\peaknet\method\jobin2.met
Date Time Collected : 08/05/03 19:43:49
System Operator : Jobin Shaeri

Vial ID :
Injection Volume : 10.00
Injection Number : 1

Peak Information : All Peaks

Peak #	Component Name	Retention Time	Amount	Peak Area	Peak Height
1		5.92	0.00	277240	28694
2		6.35	0.00	3109219	181179
3		6.93	0.00	1448086	81074
4		7.68	0.00	202438	14941
5		8.32	0.00	722137	74596
6		8.80	0.00	7536022	640359
7		9.47	0.00	1342289	100511

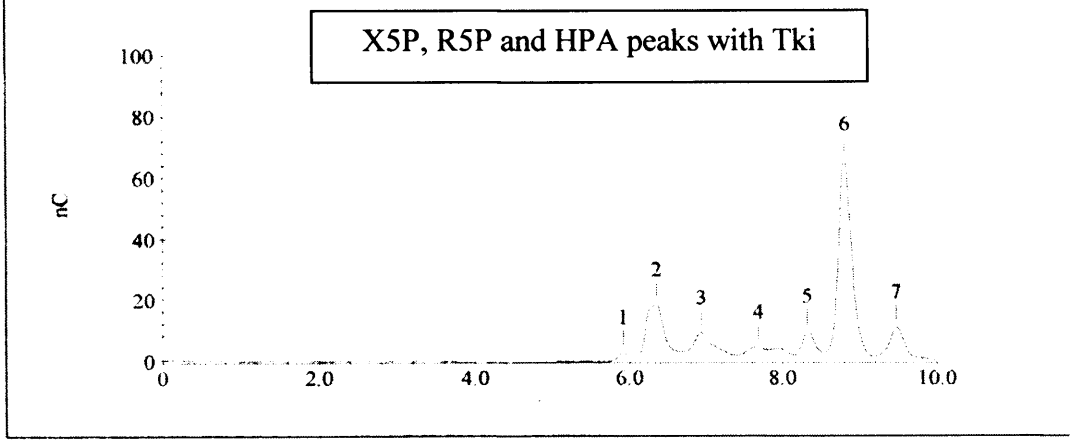


Figure I.2 X5P purification sample from DHAP reaction with Tki showing the depleted HPA (peak 5) retention time 8.32, with the product X5P (peak 2) retention time 6.35 and R5P formed (peak 6) retention time 8.80.

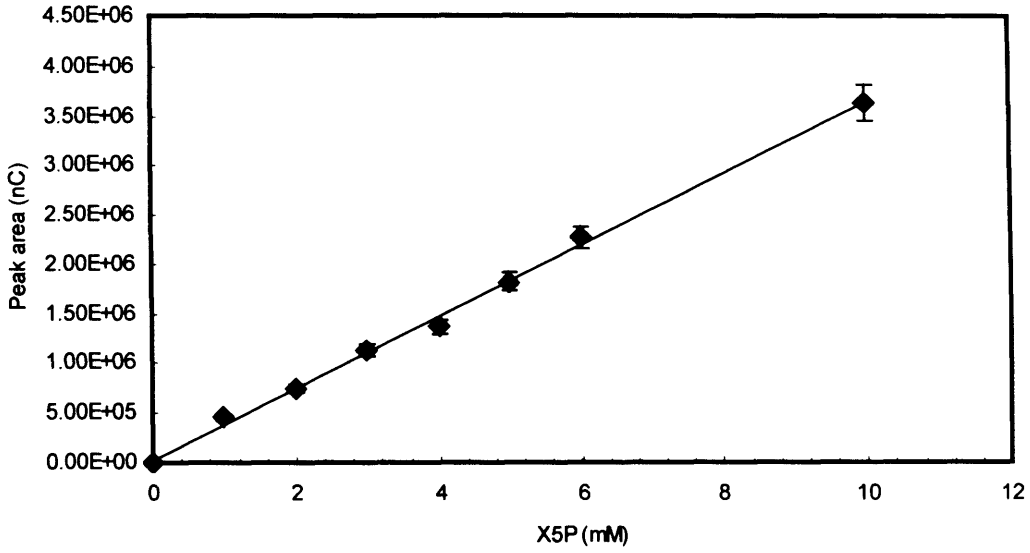


Figure I.3 X5P calibration curve from HPLC methods described in Chapter 2.

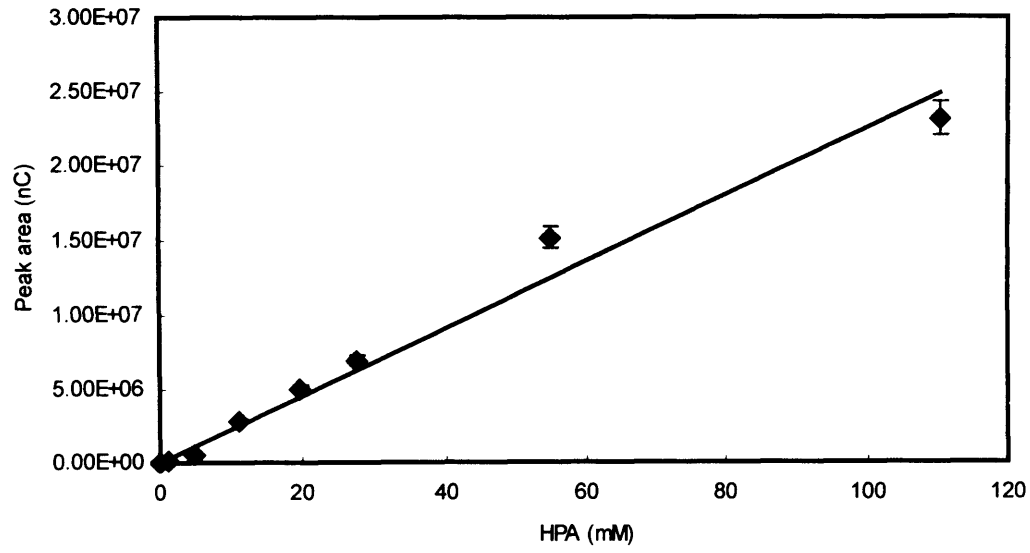


Figure I.4 A typical HPA calibration graph produced by HPLC methods described in Chapter 2.

Appendix II: Fermentations

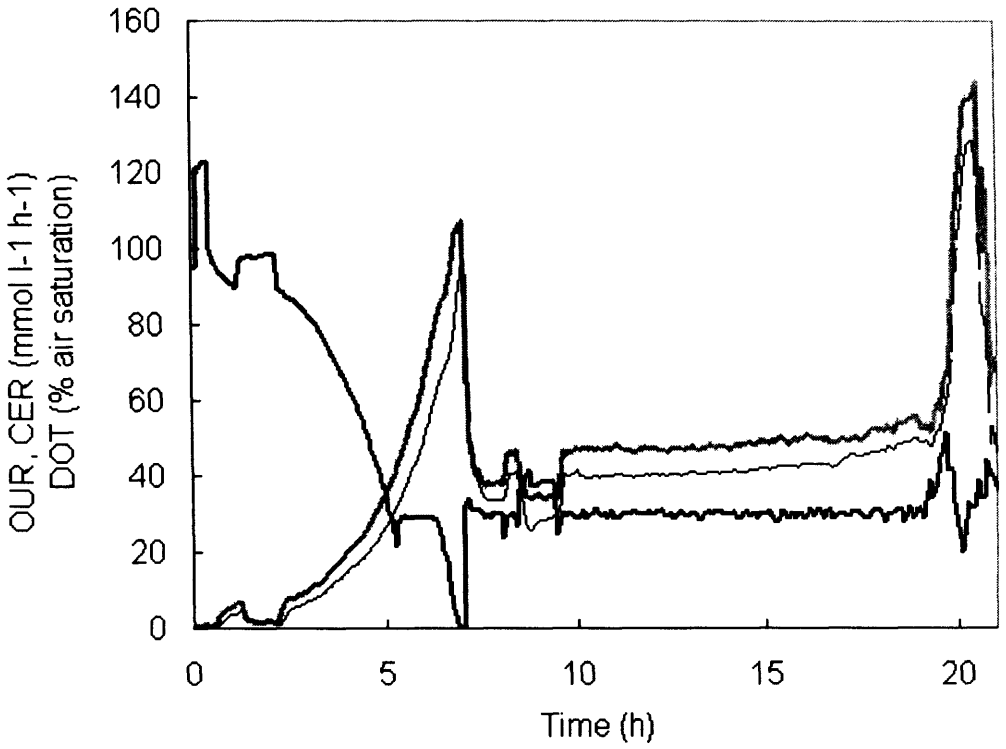


Figure II.1 A typical 50 L fed-batch fermentation profile. Figure describing DOT % air saturation (▬) OUR (▬) and CER (——).

Appendix III: OD measurements

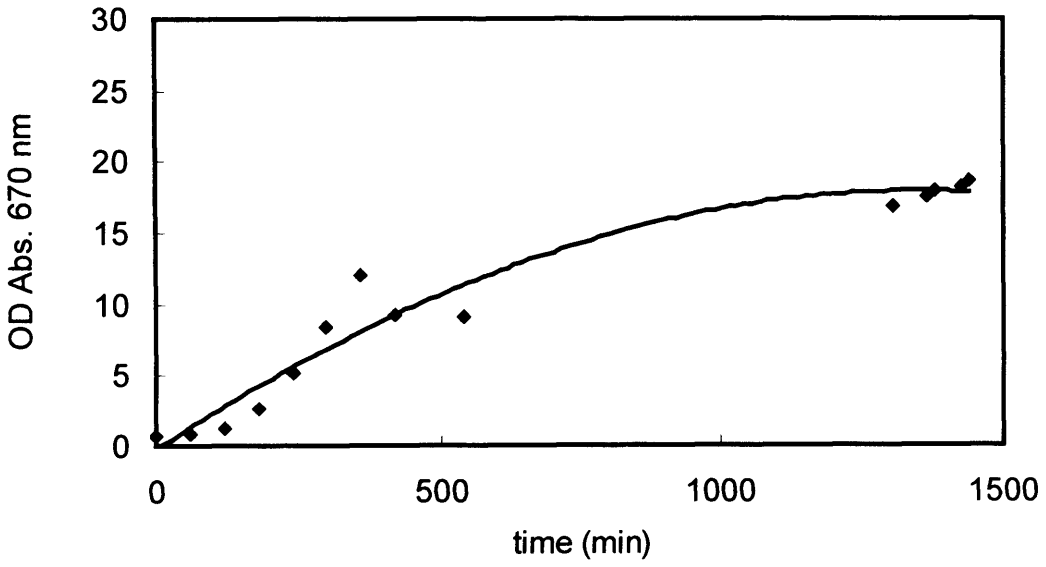


Figure III.1 OD measurements from a 50L fed-batch fermentation.

Appendix IV: DCW measurements

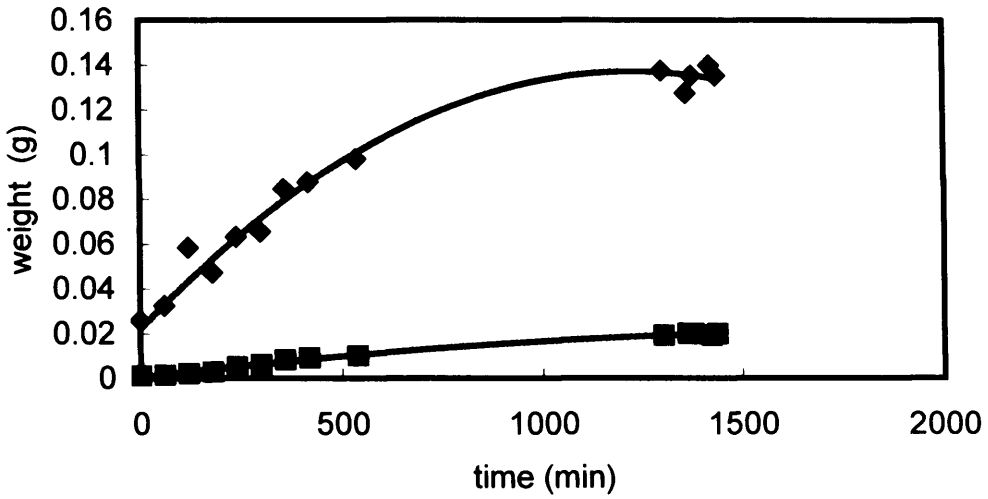


Figure IV. 1 Comparison of DCW (—■—) with wet cell weight (—◆—)

Calibration of OD_{670} with DCW

In order to determine the DCW of the *E.coli* JM107/pQR 711 cultures using the OD_{670} measurement it was necessary to calibrate the two measurements against each other. Figure IV.2 shows the calibration curve that was constructed to correlate the DCW and OD_{670}.

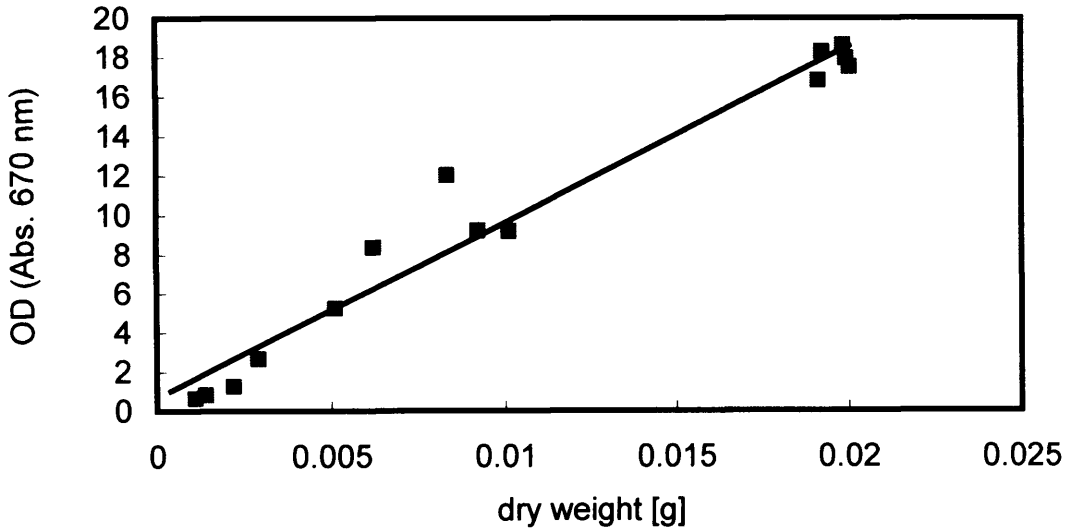


Figure IV. 2 The correlation of OD (670nm) and dry weight 9g.

Appendix V: Glycerol analysis

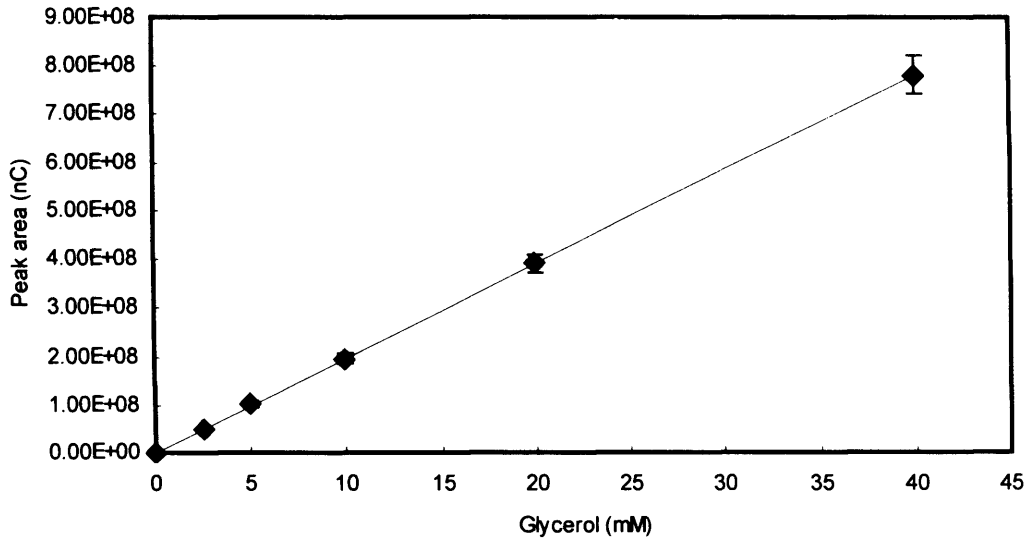


Figure V. 1 The calibration of glycerol for detection using the HPLC method described in Chapter 2. The calibration curve was used to determine glycerol concentration in the broth in the fed-batch mode of operation. Glycerol retention time was 13.27 minutes.

Appendix VI: Measurement of protein

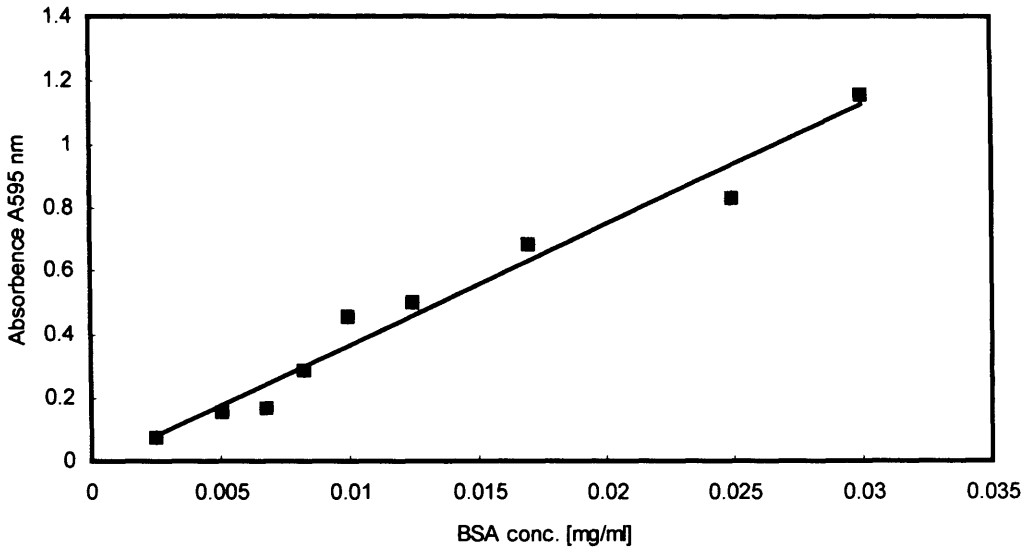


Figure VI. 1 Biorad assay calibration graph.

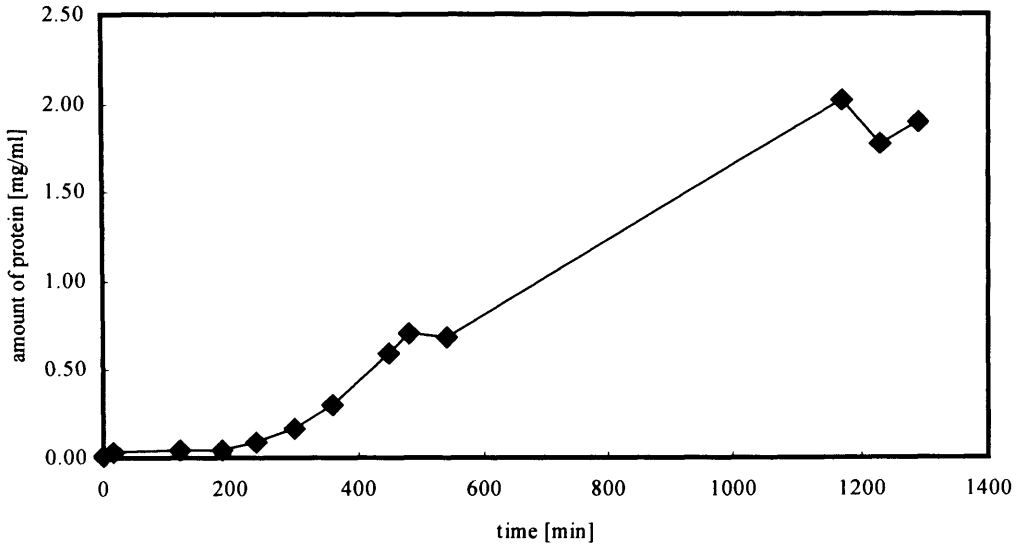


Figure VI. 2 Amount of protein with increasing time (min) in a 50 L fed batch fermentation.

Appendix VII: Transketolase activity calculations

Transketolase was calculated based on the concentration of erythrulose produced in the assay described in Chapter 2.

Example calculation:

Erythrulose concentration from HPLC = 2.35 mg/mL

2.35 mg = 19.56 μmoles produced in 20 minutes.

Activity = 0.98 μmoles/min/mL (0.98 U mL^{-1})

Based on Biorad assay:

Specific activity = (0.98 UmL^{-1})/(8mg/mL)

= 0.12 U mg^{-1}

Method File Name : c:\peaknet\method\hydroxy.met
Date Time Collected : 28/08/03 16:19:08
System Operator : Jobin Shaeri

Vial ID :
Injection Volume : 1.00
Injection Number : 3

Peak Information : All Peaks

Peak #	Component Name	Retention Time	Amount	Peak Area	Peak Height
1		1.93	0.00	1596501	49874
2		6.08	0.00	473614	68083
3		6.95	0.00	16314723	641452
4		7.98	0.00	327739	28441
5		8.48	0.00	98952099	5674827
6		10.10	0.00	345083	32144
7		10.75	0.00	4420093	281846
8		11.53	0.00	111054354	6361999
9		12.35	0.00	39779232	2378875
10		13.28	0.00	43437587	2405485
11		14.53	0.00	4882606	177430

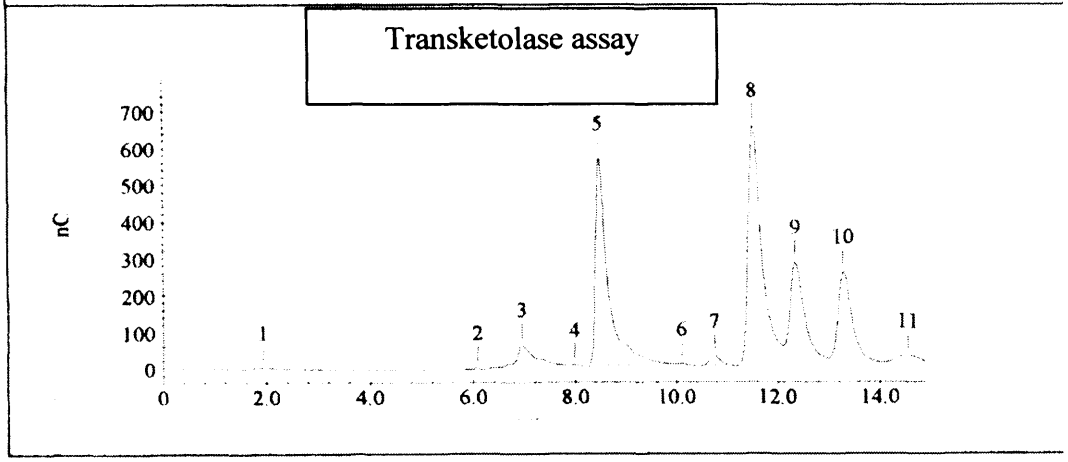


Figure VII. 1 The HPLC transketolase assay showing the peaks for HPA (retention time 8.48, peak 5), Erythrulose (retention time 11.3, peak 8) and glycolaldehyde (retention time 12.35, peak 9). The reaction sample also contained glycerol as it was a culture sample (retention time 13.27, peak 10).

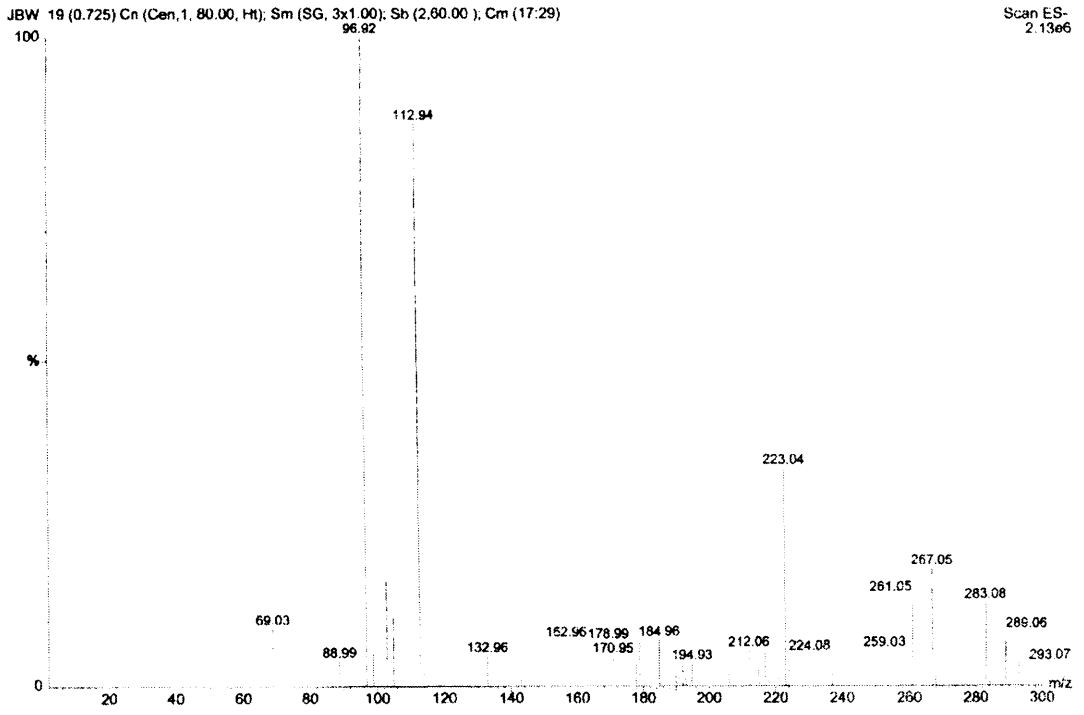


Figure VIII.1 NMR analysis (ES-) of sample containing by-product 1 illustrating the abundance of each ion (the most abundant at 100%) with increasing mass (m/z).

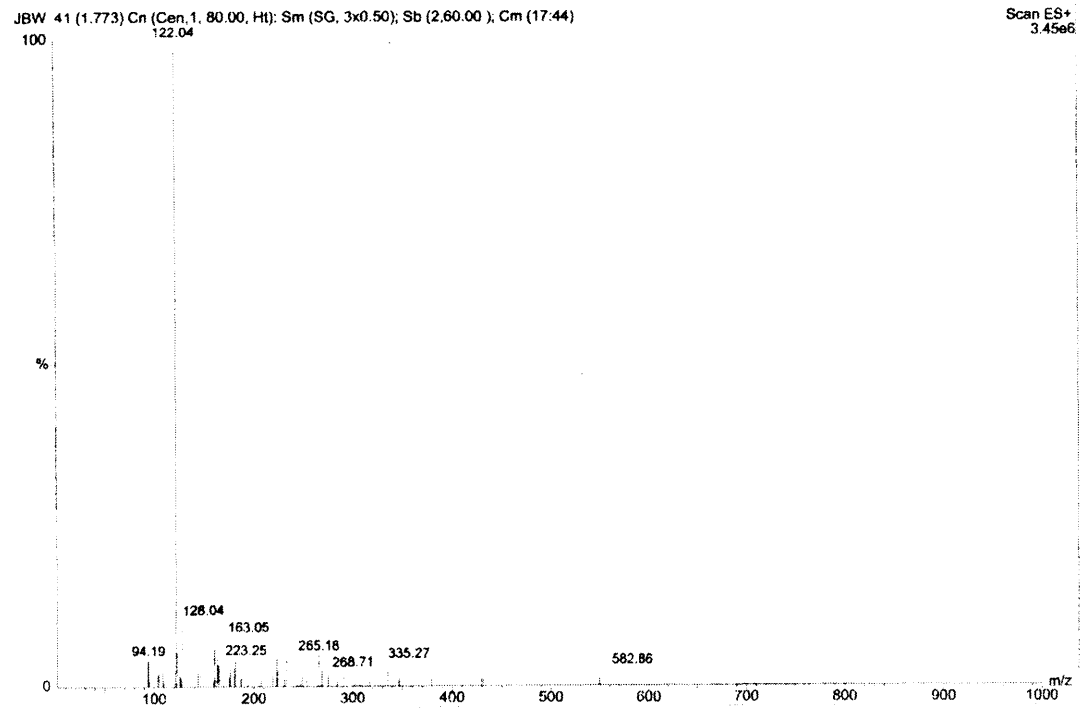


Figure VIII.2 NMR analysis (ES+) of sample containing by-product 1 illustrating the abundance of each ion (the most abundant at 100%) with increasing mass (m/z).

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